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AD_____

GRANT NUMBER DAMD17-96-1-6096

TITLE: Nitric Oxide in Mammary Tumor Progression

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REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Annual (1 Jul 97 - 30 Jun 98)

4. TITLE AND SUBTITLE
Nitric Oxide in Mammary Tumor Progression

5. FUNDING NUMBERS
DAMD17-96-1-6096

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Western Ontario
London, Ontario, Canada N6A 5C1

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Jul 98). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Nitric Oxide (NO) is a potent bioactive molecule produced in the presence of endothelial (e), neuronal (n) or inducible (i) types of NO synthase (NOS) enzymes. We had earlier shown that treatment with NOS inhibitors reduced tumor growth and metastasis of C3H/HeJ murine mammary carcinomas, mitigated IL-2 therapy-induced capillary leakage and improved antitumor effects of IL-2. These results suggested that NO promoted mammary tumor progression and mediated IL-2 therapy-induced capillary leakage. Present proposal was to identify the underlying mechanisms for the above. Results to date reveal that eNOS expression by tumor cells is positively associated with metastasis in spontaneous C3H/HeJ mammary tumors and their clonal derivatives differing in their ability for spontaneous lung metastasis; that endogenous and induced NO promoted invasiveness of the highly metastatic C3L5 mammary tumor line owing to an upregulation of matrix-degrading enzyme matrix metalloprotease (MMP)-2 and downregulation of MMP inhibitors TIMP-2 and TIMP-3; that endogenous NO promoted C3L5 mammary tumor angiogenesis in a tumor-angiogenesis model devised in this laboratory. Thus NOS inhibitors may have a valuable role in cancer therapy.

14. SUBJECT TERMS				15. NUMBER OF PAGES
Breast Cancer	Nitric Oxide	invasion		125
metastasis	C3H/HeJ mammary tumor	angiogenesis		16. PRICE CODE
IL-2 therapy				

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

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TABLE OF CONTENTS

5. INTRODUCTION

This project has been designed to explore the role of nitric oxide (NO) in mammary tumor progression, using a C3H/HeJ mouse mammary tumor model developed in our laboratory. This model employs spontaneous tumors as well as their clones which vary in their ability for spontaneous metastasis.

Biology of NO

Following the discovery (1) that NO accounts for the full biological activity of a factor initially named "endothelium-derived relaxing factor" (2), produced by endothelial cells and causing vasodilation, research on the biology of NO has grown exponentially for many years. This molecule has since been shown to be produced by many other cells in the body, providing additional physiological functions such as inhibition of platelet aggregation, modulation of neurotransmission and mediation of cytotoxic function of macrophages against microbes, parasites and tumor cells (3-8). Sustained high levels of NO produced at the sites of inflammation can also mediate pathological injuries (9).

NO is produced by the conversion of the amino acid L-arginine to L-citrulline by a family of enzymes known as NO synthases (NOS). Three isoforms of NOS have been identified so far: endothelial type or eNOS is a constitutive form present in endothelial cells, myocardial cells and other cells inclusive of certain tumor cells; neuronal type or nNOS is also a constitutive form present in the central nervous system neurons, cells of the myenteric plexus, skeletal muscle cells, renal, bronchial and pancreatic islet cells as well as in tumors of the central nervous system; inducible type or iNOS is usually induced by certain inflammatory cytokines (e.g. IFN-γ, TNF-α) or bacterial products (e.g. LPS) in macrophages, hepatocytes, chondrocytes, endothelial cells and certain tumor cells (10-14). The constitutive forms are Ca^{++} and calmodulin-dependent whereas the inducible form is Ca^{++} and calmodulin-independent. Genes for all the isoforms have been cloned in numerous species (15,16) and disrupted in mice to show that none of the disruptions were embryo-lethal but had pathological effects consistent with known biological functions of NO. For example, eNOS knockout mice are hypertensive (17) because of the loss of vaso-relaxant function of NO; iNOS knockout mice are susceptible to infection and show poor macrophage cytotoxicity against parasites and tumor cells (18), consistent with NO-mediated macrophage defense; nNOS knockout mice (19) show hypertrophic pyloric stenosis, consistent with NO-mediated relaxation of pyloric sphincter muscle. nNOS - deficient males, in addition, show abnormal sexual behavior (20) because of aberrant neurotransmission.

NO is a free radical capable of crossing the cell membrane and reacting with other molecules. Most physiological functions of NO are mediated by increases in intracellular cGMP (21,22), whereas antibacterial, antiparasitic and antitumor functions of macrophage-

derived NO have been ascribed to the inhibition of mitochondrial respiration and DNA synthesis in target cells (23).

Constitutive production of NO occurs in cells at low to moderate levels, and the resulting bioactivity is short lived (T½ = few seconds) and short-range in nature. On the other hand, induced production of NO can be sustained at high local levels for a longer duration if the inducer molecules, e.g. inflammation-associated cytokines are produced in a protracted manner. This often leads to pathological consequences, resulting from NO reaction products. NO reacts with molecular oxygen, transition metals and superoxide to form intermediates which can cause cellular injury. For example, NO reacts with superoxide to make peroxynitrite, which can cause DNA damage (24).

Role of NO in tumor progression

It has been recognized for some time that chronic NO production is genotoxic and thus potentially carcinogenic (24). Recent studies, including our own (25, Appendix 1) have revealed that tumor or host-derived NO can profoundly influence tumor progression in a positive or negative manner depending on the circumstances, and that in a large panel of well-established tumors, which have been examined so far, NO usually promotes tumor progression. Elevated serum NO levels have been observed in many cancer patients (26) indicating that tumor cells or host cells serve as the additional source of NO in these patients. A high expression of active NOS enzymes in tumor cells (27,28,31,32,32a), endothelial cells in tumor vasculature (28) or tumor-infiltrating macrophages (29,30,32) has been positively correlated with the degree of malignancy in human cancers involving a large number of tissues: cancers of the reproductive tract (uterus, ovary) (27), central nervous system tumors (28), breast cancer (29), gastric cancer (30), cancer (squamous cell carcinomas) of the head and neck (31), prostate cancer (32) and lung cancer (33). However, the underlying mechanisms remain unexplored. Unexpectedly an inversion of this relationship was reported for human colonic tumors (34,35); this finding was in contrast with another study reporting that many human colon cancer cell lines exhibited significant NOS activity (36). This discrepancy has finally been resolved in a recent study (37) showing that the highest expression of active iNOS was noted in human colonic adenomas prior to their progression into carcinomas, consistent with the hypothesis that this promoted the transition of adenomas into carcinomas by a stimiulation of angio-genesis. A positive correlation between NOS expression or NO production and tumor progression has also been detected in experimental tumor models in the mouse (38) and the rat (39).

A direct evidence for a stimulatory role of NO in tumor progression came from our own findings in a murine mammary adenocarcinoma model (40,41) that treatments with either of two NOS inhibitors N^G-methyl-L Arginine (NMMA) and N^G-nitro-L-Arginine methyl ester (L-NAME) reduced the growth of the primary tumors and their spontaneous lung

metastases in mice transplanted with the C3L5 mammary tumor line (see figures 3, 4, 5, 6, in Appendix 1). Similar findings were reported with L-NAME therapy in a rat colonic adenocarcinoma model (39). In support of these results, engineered expression of iNOS in a human colonic adenocarcinoma line resulted in an increased growth rate and vascularity of tumors following transplantation in nude mice (42). In contrast with these results, engineered overexpression of iNOS in an iNOS deficient murine melanoma line (43,44) or a human renal carcinoma line (45) suppressed tumorigenic and metastatic ability of tumor cells *in vivo* because of NO-mediated cytostasis and apoptosis (43,44). Two explanations may be offered for these apparently conflicting results: First, very high NO levels (such as those produced by the iNOS-transduced murine melanoma line) (43,44) can be detrimental to tumor cell survival; for example the iNOS-overexpressing melanoma line had poor survival in the absence of NOS inhibitors *in vitro* and *in vivo* (44). Second, tumor cells may vary in their susceptibility to NO-mediated cytostasis and apoptosis because of their genetic makeup. For example, it has been suggested that the functional status of the tumor suppressor gene p53 dictates susceptibility (if functional) or resistance (if non-functional) to NO-mediated cytostasis or apoptosis (46,47). This suggestion was based on the following findings: iNOS transfected tumor cell lines fell into two distinct categories. Those expressing functional wild type p53 were vulnerable to NO-mediated cytostasis because of an accumulation p53 protein induced by endogenous NO (46,47). On the other hand, tumor cells in which p53 gene was lost or mutated not only withstood the deleterious effects of endogenous NO, but also exhibited faster growth and vascularity when transplanted *in vivo* (47). Since p53 mutation occurs in nearly half of human cancers (48), it was hypothesized that NO would facilitate tumor progression in a large proportion of well-established human tumors (47). We hypothesize that during the clonal evolution of tumors *in vivo*, high NO producing clones susceptible to NO-mediated injury are deleted and selected against those which are genetically resistant to NO-mediated injury and capable of utilizing NO to their advantage for expression of an aggressive phenotype (25, Appendix 1). Loss of functional p53 gene may represent one of many genetic changes which can possibly result in the above phenotype. Further studies are needed to identify other genotypic markers in tumors for susceptibility or resistance to NO-mediated injury, so that the information can be utilized in therapeutic designs.

C3H/HeJ mammary tumor model employed in the present project.

Details of this model are provided in ref. 25 (Appendix 1). In brief, this model is a combination of spontaneous C3H/HeJ mammary tumors and some of their clonal derivatives produced in our laboratory. Approximately 90% of retired breeder females of this mouse strain spontaneously develop invasive mammary adenocarcinomas with a pseudoglandular architecture, most of which metastasize to the lungs. This is due to insertional mutagenesis of certain cell growth-regulating loci resulting from the integration of the proviral form of the mouse mammary tumor virus (MMTV) in the developing

mammary tissue of mice receiving the virus via mother's milk. Approximately 39% of human breast cancer specimens express a 660 bp sequence of the MMTV envelop gene (49), the epidemiological significance of which remain to be identified. This finding and the similarity in histological features suggest that C3H/HeJ spontaneous mammary tumors may represent the closest model for the human breast cancer, in particular, the familial form. We have derived two clonal lines, C3L5 and C10, grown from a spontaneous mammary tumor-derived line T58. The metastatic phenotype for C3L5 is high, for C10 is low, and for T58 is intermediate, based on the number of spontaneous lung metastases from subcutaneously transplanted tumors.

Preliminary data provided in the original grant application and substantiated further in last year's annual report revealed that spontaneous C3H/HeJ primary tumors expressed eNOS protein (based on immunocyto-chemistry) in a heterogenous manner in tumor cells, whereas their metastases in the lungs were uniformly and strongly positive for eNOS (Figure 1, Appendix 1). This finding suggested that eNOS bearing cells in the primary tumor were more prone to metastasis. This suggestion was strengthened by the findings that C3L5 cells (highly metastatic) were strongly positive for eNOS *in vitro*, as well as *in vivo* both at primary and metastatic sites (Figures 1 and 2, Appendix 1). In addition, iNOS was inducible in C3L5 cells when cultured with IFN-γ and LPS (Figure 2, Appendix 1). In contrast, C10 cells (poorly metastatic) were weakly positive for eNOS, and the expression was heterogenous. These findings, combined with our published observations (40,41); see also figures 3, 4, 5 and 6 in Appendix 1) that two NOS inhibitors NMMA and L-NAME reduced the growth of C3L5 primary tumors as well as their spontaneous lung metastases, led us to hypothesize that tumor-derived NO promoted tumor progression in this mammary tumor model. A large component of the current project is to validate this hypothesis and to identify the mechanisms underlying NO-mediated promotion of tumor progression in this model.

Role of NO in "capillary leak syndrome"

We have discovered that capillary leak syndrome (characterized by fluid leakage from the capillaries into tissue spaces, various organs and body cavities), a life-threatening side effect of interleukin-2 (IL-2) based cancer immunotherapy, is due to the increased production of nitric oxide (50, 51). This was shown by (a) a positive correlation of NO levels in the serum and the body fluids with the severity of IL-2 therapy-induced capillary leakage in healthy and tumor-bearing mice, and (b) an amelioration of this capillary leakage by chronic oral administration of NOS inhibitors NMMA and L-NAME (50-53, see Appendix 2 for a comprehensive summary).

Unexpectedly, we also observed that additional therapy with NOS inhibitors improved antitumor/antimetastatic effects of IL-2 therapy (50,53). This finding led to the suggestion that NO induction by IL-2 therapy interfered with antitumor effects of IL-2

therapy. We tested this hypothesis by investigating the effects of addition of L-NAME on IL-2 induced generation of lymphokine activated killer (LAK) cells *in vivo* and *in vitro* in healthy and tumor bearing mice (54). Results revealed that inhibition of NO production *in vivo* or *in vitro* by addition of L-NAME to IL-2 therapy or IL-2 induced lymphocyte activation *in vitro* caused a substantial enhancement of LAK cell activation. In other words, IL-2 induced NO production interfered with optional LAK cell activation which can be abrogated with NOS inhibitors (54).

A minor component of the current project was to (a) identify the cellular source of NO induced by IL-2 therapy, (b) identify the nature of structural damage to the lungs of mice suffering from IL-2 induced pulmonary edema and pleural effusion, and (c) examine the effects of L-NAME therapy on the above parameters. Results of these studies were reported in last year's annual report and have been published (52). In brief, IL-2 therapy led to highl levels of iNOS protein expression and activity in the tissues of the anterior thoracic wall in accompaniment with pleural effusion. There was structural damage to the lungs (alveolar epithelium and interstitial tissue) and its capillaries by IL-2 therapy, which were mitigated by L-NAME therapy. L-NAME therapy abrogated IL-2 induced rise in iNOS activity but not the expression iNOS protein in the tissues.

6. BODY OF THE PROGRESS REPORT

Overall Hypothesis: Tumor derived NO promotes C3H/HeJ mammary tumor progression and metastasis.

Overall Objectives:

(1) To validate the hypothesis of the stimulatory role of NO in mammary tumor progression by further correlation of eNOS expression with metastasis, and investigating the effects of down-regulating eNOS gene on tumor growth, angiogenesis and metastases.

(2) To identify mechanisms of NO-mediated stimulation of tumor progression by investigating the role of NO in tumor cell proliferation, invasiveness and angiogenesis.

Our assessment of overall progress in relation to the statement of objectives

Task 1 **Relationship between NOS expression and tumor progression/ metastasis:** Progress has matched with our expectations. The molecular biology component was frustrating at the beginning because of our failure to knockout the eNOS gene in C3L5 cells. We have since recognized that this is possibly because of increased number (3.6) of gene copies in these cells, and adopted the antisense approach to downregulate eNOS. We have made satisfactory progress during the last year.

Task 2 **Identification of mechanisms of tumor progression by NO.** Although this task was initially assigned to Year II onwards, we have achieved significant progress in this area within Years I and II.

Task 3 **Mechanisms underlying IL-2 induced capillary leakage and interference with antitumor effects of IL-2 therapy by IL-2-induced NO.** Although this task was initially assigned to Year III onwards, significant progress has already been achieved in this area. We shall conduct some newer experiments to be designed on the basis of newer knowledge. (See later).

Record of Research findings during the current year.

Task 1 Relationship between NOS expression and tumor progression and metastasis.

(a) Relationship between the expression of NOS protein and tumor growth and metastasis.

(i) Spontaneous C3H/HeJ mammary tumors. We have now expanded and validated our earlier data (presented in last year's annual report) in another 10 spontaneous mammary tumors.

Materials and Methods:

Ten new C3H/HeJ spontaneous tumors were harvested from C3H/HeJ retired breeder females at 8-12 weeks of tumor age. This gave us a total of 20 spontaneous tumors to date. Based on the growth rate of primary tumors, four had fast growth rate, eleven had intermediate growth rates and five had slow growth rate. The latter tumors showed some spontaneous regression which occurs in about a quarter of the tumors as reported by us earlier (55). All animals showed lung metastases. Histologically, all primary tumors showed variable local tissue invasiveness, however regressing tumors showed evidence of tumor cell death and extensive mononuclear cell infiltration.

Both primary and metastatic tumors (5-7 μm thick sections fixed in buffered formalin and paraffin-embedded) were permeabilized in 0.1% triton in PBS for 20 min and immunostained for eNOS and iNOS enzymes by treatment with primary antibodies (monoclonal e NOS and iNOS antibodies raised in mice, Transduction Lab, Lexington, KY) followed by biotinylated horse anti-mouse secondary antibodies and avidin-biotin conjugate (ABC) and DAB chromogen treatment. Negative controls were provided by mouse Ig replacing the primary antibodies.

Results:

All primary tumors showed tumor cells positive for eNOS. There was a good deal of heterogeneity in the distribution of eNOS positive cells within the same tumor irrespective of growth rate (40-70%, depending on the site within the tumor). The mean % eNOS positive cells did not correlate well with the growth rates of the primary tumors. However, all metastatic tumors were strongly positive for eNOS (76-95% +ve cells). iNOS positivity was not seen in primary or metastatic tumor cells. Only a certain proportion of macrophages and stromal cells showed iNOS staining. A representative micrograph is provided in figure 1 of Appendix 1.

Conclusions:

Spontaneous primary tumors were heterogenous for eNOS protein expression irrespective of growth rate but tumor cells in metastatic foci were always predominantly eNOS positive. Tumor cells did not express iNOS, but iNOS was expressed by certain macrophages and stromal cells. These results strengthen our hypothesis that eNOS expression provides a selective advantage for primary tumor cells to metastasize.

(ii) C3L5 (highly metastatic) and C10 (weakly metastatic) cell lines and their transplants.

Some of the data were presented at the "Era of Hope" meeting (appendix 5) and the last meeting of the American Association for Cancer Research (Appendix 6).

Materials and Methods:

***In vitro* studies:** C3L5 and C10 cells grown under normal culture conditions or after 24 h preculturing with LPS and IFNγ to induce iNOS protein were immunostained for eNOS or iNOS protein. The cells were briefly fixed in ice cold methanol and treated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. Cells were then incubated with 10% normal horse serum in 0.2% bovine serum albumin (BSA) in PBS to inhibit non-specific antibody binding, followed by overnight incubation with the mouse monoclonal antibodies against eNOS or iNOS protein (1:50 dilution in 0.2% BSA in PBS; Transduction Laboratories, Lexington, KY). The next day, the cells were incubated with biotinylated horse anti-mouse secondary antibodies (1:200 dilution in 0.2% BSA in PBS; Vector Laboratories, Burlingame, CA), followed by avidin-biotin conjugate and diaminobenzidene chromogen treatment, which results in a brown reaction product. The primary antibody was omitted and replaced with 0.2% BSA in PBS or a mouse IgG at a concentration equivalent to the concentration of the primary antibody to serve as negative controls.

***In vivo* studies:** Numerous experiments were conducted to examine the differences in primary tumor growth, development of lung metastases and eNOS and iNOS protein expression in the primary tumors and lung metastases developing in C3H/HeJ female mice bearing transplants of C3L5 or C10 tumors (n = 30 in both cases). Healthy 6-8 week old C3H/HeJ female mice were subcutaneously injected in the left axillary region with 5×10^5 C3L5 or C10 tumor cells. During a three week period, tumor development at the primary site of injection was monitored by measuring the maximum and minimum diameters of the primary tumor which were used to calculate tumor volume (= $0.52a^2b$, where a = min diameter, b = max diameter). Three weeks post-injection, mice were killed to harvest primary tumors

and their metastases. Lungs were inflated with Bouin's fixative to enable visualization and counting of metastases (if present) under a dissection microscope. Samples of primary tumors and lungs were fixed in 4% para-formaldehyde for paraffin sections and also snap-frozen in liquid nitrogen for measurement of NOS activity and protein and RNA isolation.

Immunohistochemistry for eNOS and iNOS protein was conducted on 7 μm thick paraffin sections of primary tumors and lung metastases, as described in the earlier section for spontaneous mammary tumors.

Results:

***In vitro* studies:** We confirmed our earlier observations that strong eNOS staining was exhibited by almost all C3L5 cells with very little heterogeneity (Figurer 2A, Appendix 1; Figure 1A, Appendix 4), whereas C10 cells showed weaker eNOS staining (approximately in 70% of cells) and more heterogeneity (Figure 1). Both C3L5 and C10 cells precultured with LPS and IFNγ exhibited moderate iNOS staining in approximately 30-50% of the cells (Figures 1 and 2B, Appendix 1).

***In vivo* studies:** The rate of primary tumor growth as given by tumor volumes during the three week period was higher in C3L5 than in C10 – injected mice (Figure 2). At 14 and 21 days after injection, C3L5 tumors were significantly larger than the C10 tumors. The number of spontaneous lung metastases was also significantly higher in C3L5 than in C10 transplanted mice (Figure 3).

C3L5 primary tumors were strongly positive for eNOS protein in approximately 80% of tumor cells and demonstrated some heterogeneity in eNOS staining. C10 primary tumors were more heterogeneous for eNOS staining and showed a lower proportion (approximately 40%) of eNOS positive tumor cells. Spontaneous lung metastases resulting from both C3L5 and C10 transplants exhibited a similar degree of eNOS positivity. Approximately 40-50% of the tumor cells in metastatic foci were eNOS positive in both cases (Figure 4).

Conclusions: We have shown that two tumor lines (C10 and C3L5) clonally derived from the same spontaneous mammary tumor significantly differed in their *in vivo* growth rates and ability for spontaneous lung metastasis. These differences were positively correlated with their differences in eNOS protein expression *in vitro* and *in vivo* only in the primary tumors. Their metastatic counterparts expressed similiar levels of eNOS protein. These results confirm our hypothesis that eNOS protein expression facilitated tumor growth and metastasis. Currently these studies are being extended to measurements of NOS activity.

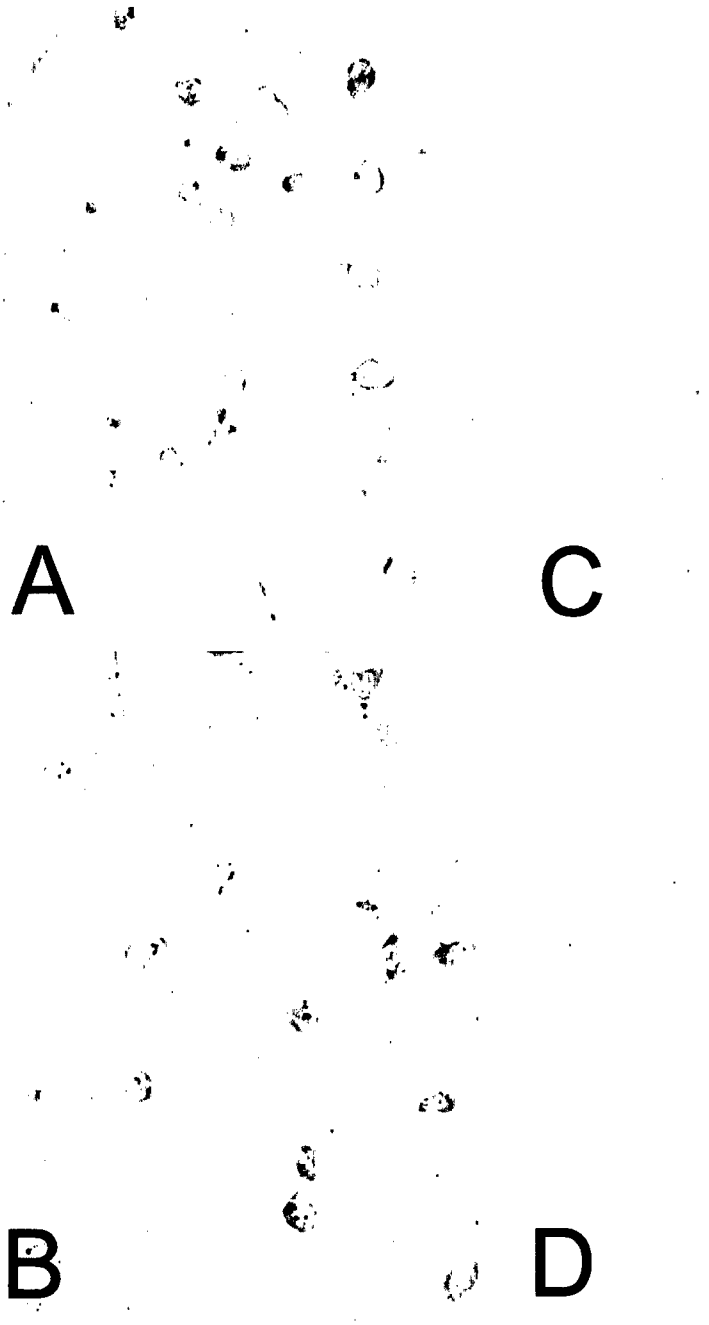


Figure 1. Immunocytochemical staining of cultured C10 tumor cells for eNOS and iNOS protein. Cells examined for iNOS protein were precultured for 24 h with LPS and IFNγ. Positive immunoreactivity is indicated by brown staining. **A**, eNOS immunostaining in C10 cells was heterogeneous and weak to moderate in intensity. Approximately 70% of the cells showed positive staining. **B**, Approximately 30-50% of C10 cells demonstrated moderate iNOS positivity induced by LPS + IFNγ. **C & D,** Negative controls for eNOS and iNOS staining of cultured C10 cells, respectively.

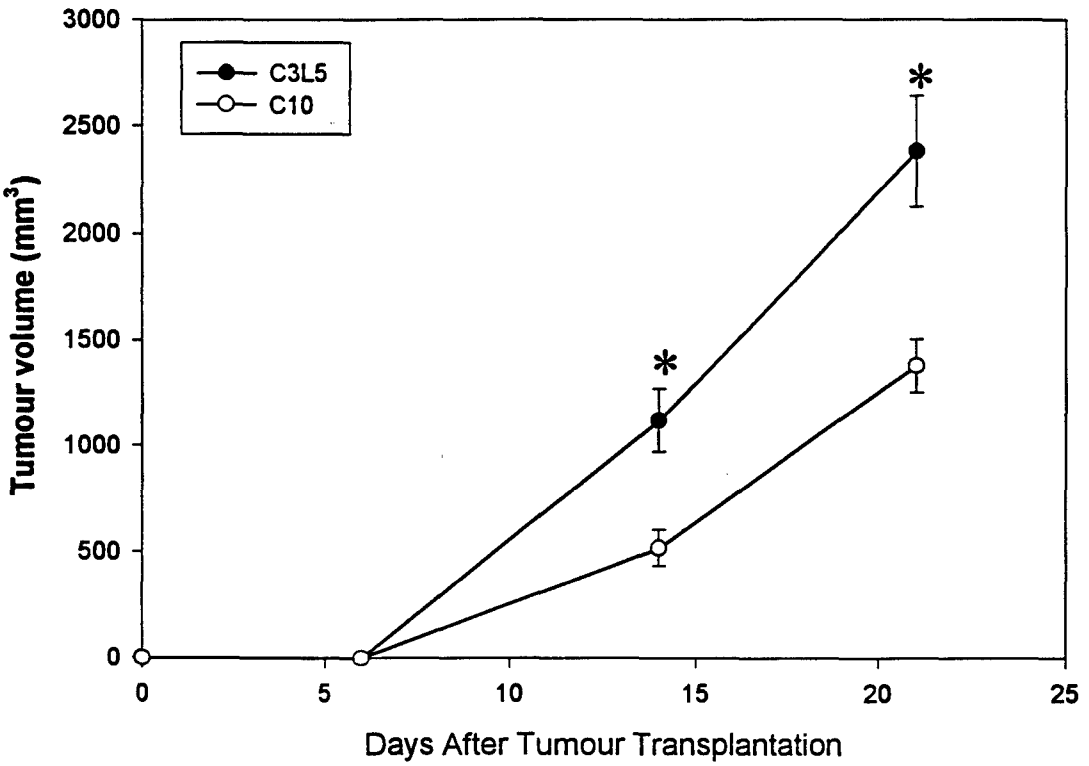


Figure 2. Volumes of primary tumors growing in C3H/HeJ mice subcutaneously injected with either C3L5 or C10 tumor cells. 5×10^5 cells in 0.5 ml RPMI medium were injected in the axillary region of each mouse. Minimum and maximum tumor diameters were measured at various intervals and tumor volume was calculated as $0.52\ a^2b$, where a is the minimum diameter and b is the maximum diameter. (n = 15/group; t-test, $^*P = 0.002$)

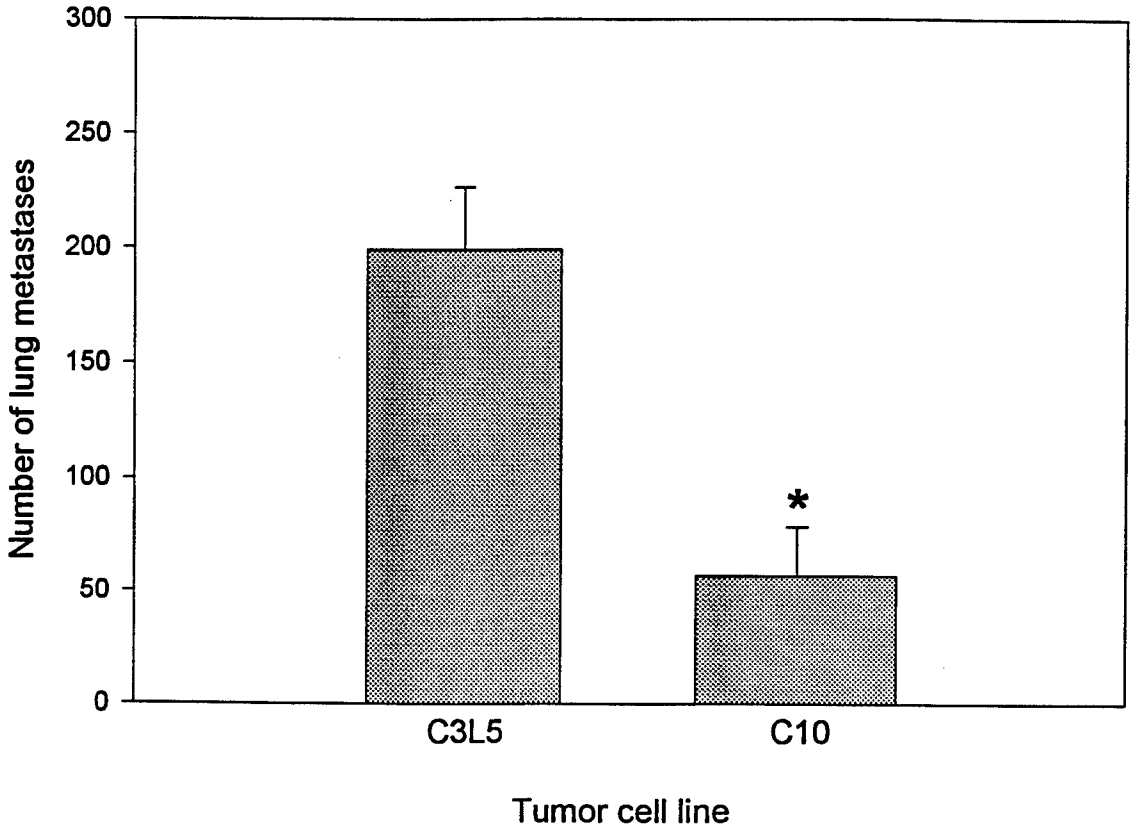


Figure 3. Mean (± standard error) number metastatic nodules in both lungs from C3H/HeJ mice 3 weeks after subcutaneous injection of 5 x 10^5 C3L5 or C10 tumor cells. (n = 15/group; Mann-Whitney Rank Sum test, $^*P < 0.001$).

Figure 4. eNOS immunostaining in 3 week old primary tumors and lung metastases in C3H/HeJ mice bearing C3L5 and C10 tumor transplants. Positive immunoreactivity is indicated by brown staining. Sections were counterstained with hematoxylin. **A**, Subcutaneous C3L5 primary tumor exhibiting eNOS positivity in approximately 80% of tumor cells. **B**, Subcutaneous C10 primary tumor exhibiting heterogeneous eNOS positivity in approximately 40% of tumor cells. Note that endothelial cells of the tumor vasculature are eNOS positive in both cases. **D & E,** Heterogeneous eNOS staining is seen in both the C3L5 (**D**) and C10 (**E**) tumor cells at the sites of respective lung metastases. Approximately 40-50% of tumor cells exhibit eNOS positivity in both cases. **C & F**, Negative controls for eNOS staining of C3L5 primary and metastatic tumors.

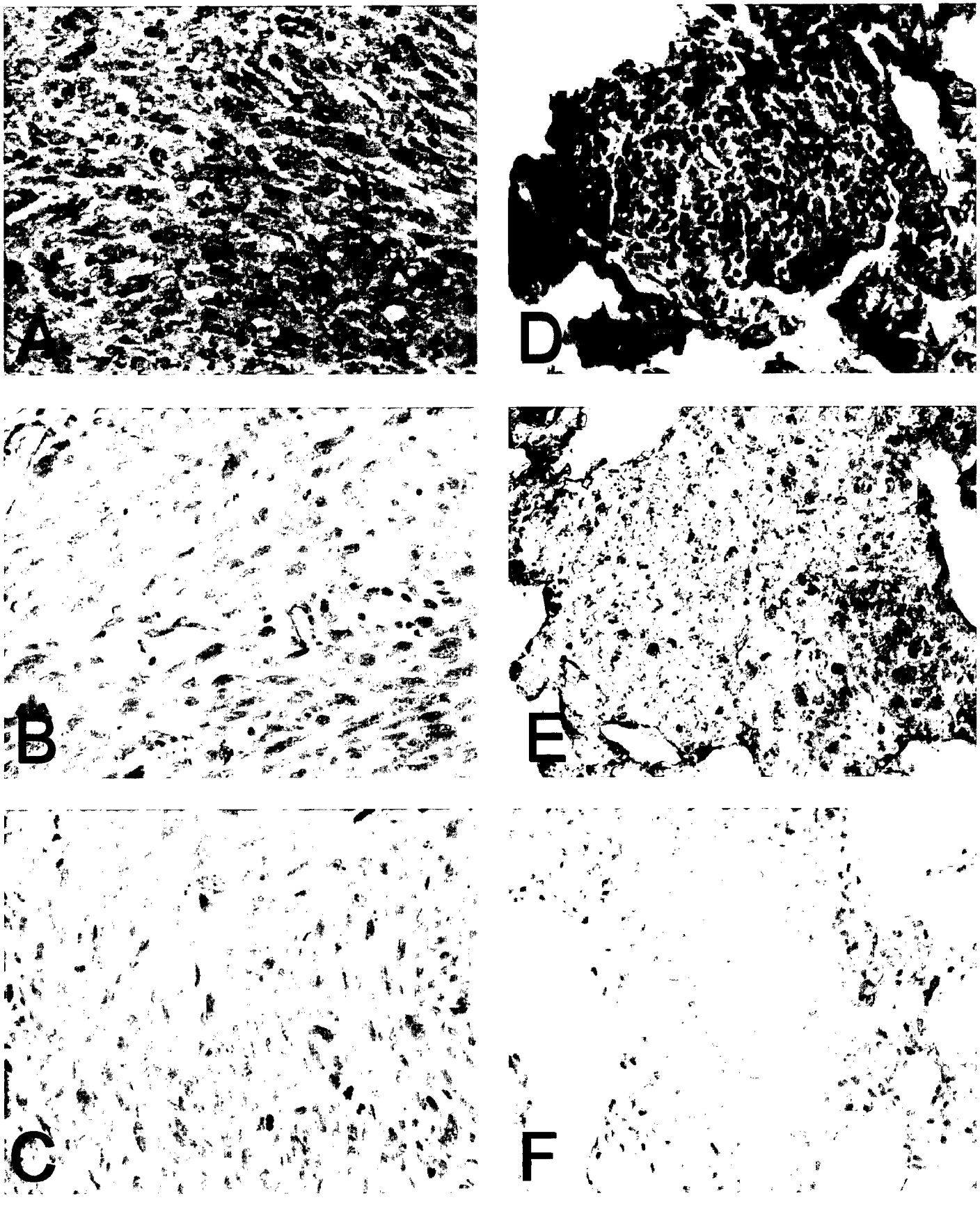


Figure 4

(b) Attempts to investigate biological alterations of murine mammary carcinoma cell line(C3L5) caused by downregulation of eNOS gene expression in these cells.

Methods and Results:

We have indicated in the last years' report that we failed to knockout eNOS gene in C3-L5 breast cancer cells. While exploring the reasons for the failure, we found, by karyotype analysis(presented in the last year's annual report) that these cells contain a modal number of 56 chromosomes, i.e. 16 chromosomes more than nornal mouse somatic cells. This raised the possibility of greater than diploid copy number of the eNOS gene in these cells, and prompted us to analyze the average copy number of this gene. This was done by Slot-blot analysis using 32P-labelled 2.4kb fragment of mouse eNOS cDNA (kindly provided by Dr. Phillip Marsden, University of Toronto). The results in triplicate were analyzed by densitometry using Mocha program. The copy number was calculated to be 3.47±0.57; and it is well known that deletion of a gene having more than two copies is very difficult. Therefore, instead of trying to knock-out, we decided to try to knock down the eNOS gene using the "antisense" approach.

First, we examined eNOS mRNA expression in C3-L5 cells by RT-PCR. Briefly, we isolated total RNA with Trizol reagent (GIBCO) from cultured C3-L5 cells and amplified them with "SuperScript One-step RT-PCR System" from GIBCO Life Technology. Specific primers complementary to murine eNOS cDNA(Genbank accessory number U53142) were used:

5'-end sense primer 5'-AGCTGGCATGGGCAACTTGAA-3' (bases 1-21)
3'-end antisense primer 5'-GTGAACATTTCCTGTGCAGT-3' (bases 611-630)

They were synthesized to amplify a 630 bp product. The products were then fractionated with 2% Argrose gel electrophoresis, and the expected 630-bp cDNA band was visible by ethidium bromide staining under UV light as shown in Figure 5A. To verify the specificity of the amplified products, we transferred the gel to a Nytran Plus membrane and hybridized it with ^{32}P-labeled mouse eNOS cDNA probe (as the above). The phosphoimage of the blot in Fig 5B demonstrated that the amplified band is eNOS specific.

We have subcloned a 4kb full length cDNA of human eNOS to a mammalian expressing vector pCR™-3 at both sense and antisense orientations. This plasmid is driven by a CMV promoter with the multicloning sites and a SV40 polyA signal as seen in Figure 6. The unique restriction enzyme sites for identification of the directions of insertion of cDNA were located with the assistance of a software from genetic computer group (gcg). The reconstructed plasmid DNAs were identified by restriction enzyme digestion and running on 1% Argrose gel electrophoresis (Figure 7). C3-L5 cells were transfected with

the plasmids of human eNOS cDNA at either sense or antisense directions in the presence of LipofecAMINE and selected by the inclusion of G418 at the concentrations of 400 μg/ml to 800 μg/ml. Twenty-four G418-resistant cell colonies have been selected from both antisense and sense transfected C3-L5 cells after two weeks exposure of G418. They are being tested by RT-PCR analysis, immunocytochemistry, and NOS activity assay. Figure 8 shows some results of RT-PCR in sense and antisense transfected clones of C3L5 cells. The finally selected cell colonies (i.e. those antisense transfected cells which will show down-regulation of eNOS at least upto 6 months) will be tested for their biological activities, e.g. invasiveness (by matrigel invasion assay), metastasis and angiogenesis, as compared to untransfected, mock-transfected or sense-transfected cells.

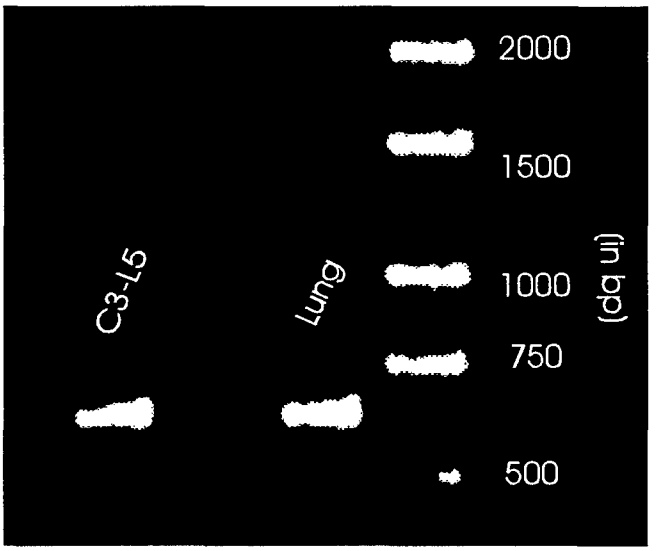
It is also well known that the antisense oligonucleotide knock-down strategy offers the unique potential to be a highly selective tool for arresting gene transcription and mRNA translation into functional protein. Therefore, we have simultaneously taken this strategy in order to knock-down eNOS gene in C3-L5 cells. We have focused on four major sites along the murine eNOS cDNA gene with a highlight at 3'-UTR which has been documented to block targeted gene expression more efficiently by an RNase H-mediated degradation process (56). However, since no information about the 3'-UTR of mouse eNOS cDNA was available, we have amplified and sequenced a 182-bp 3'-UTR fragment of eNOS cDNA. In order to amplify 3'-UTR of mouse eNOS cDNA by RT-PCR, we synthesised (PE Applied Biosystems) several primers based on the information available from Genbank to hunt for the 3'-UTR sequence of mouse eNOS. The primers used in this experiment contained one sense primer of 5'-TACCTGGTTCCTGACAGTCT-3' (bases 3603-3622 of mouse eNOS cDNA Genbank accession # U53142) and two antisense primers which included the antisense primer-1: 5'-AGCCTCTGGACAGATGTGAG-3'(bases 3761-3980 of human eNOS cDNA Genbank accession # M93718) covering an over 120-bp after the stop codon in human eNOS cDNA; antisense primer-2: 5'-GTAGTTCTCCTAACATCTGG-3' (bases 4037-4057, covering a fragment of near 400-bp based in human eNOS cDNA after the stop codon). Another set of primers used for amplification of 3'-UTR was the sense primer 5'-GGATCAGCAACGCTACCAGACCACATT-3' (bases 3454-3481 of murine eNOS cDNA Genbank accession #U53142) and the antisense primer 5'-GGCATCTTAGTAGGTCTCCTAACTT C-3' (bases 4044-4069 of bovine eNOS cDNA Genbank accession # M95674) in an attempt to produce a cDNA fragment covering 166 bases of coding region and 393 bases of 3'-UTR of eNOS according to the bovine eNOS cDNA sequence. Among these attempts, only a 182-bp cDNA fragment was amplified with the set of sense primer (bases 3603-3622) and antisense primer-1 (Figure 9). The amplified cDNA fragment was then used as a template in the next PCR to produce enough material for sequencing. The PCR product was sequenced with dRhodamine Terminator Cycle Sequencing kit and read by ABI Prims 377 DNA sequencer. This sequence (presented in Chart 1) showed no homology with any other genes as searched by gene bank.

The oligonucleotides we designed in order to inhibit eNOS gene expression in C3-

The oligonucleotides we designed in order to inhibit eNOS gene expression in C3-L5 cells have included the sequences of 5' end region with start codon, 3' end region with stop codon, eNOS gene coding regions against Calmodulin domain and NADPH-A domain but with no homology in gene sequence with other species or tissues, and three oligos against 3'-Untranslated Region(3'-UTR). For each of the antisense oligonucleotides, we designed sense and mismatched oligos as controls. Chart 2 shows the sequence details for these oligo's. All of the antisense oligonucleotide sequences listed in Chart 2 have been sent to the ISIS Pharmaceuticals for the synthesis with methoxy-ethoxy modifications, because these modifications were reported to confer the oligos a resistance to the enzymatic degradation and thus provide a reasonably longer life within the transfected cell (up to 2 weeks) for the gene knock-down experiments. Two sets of antisense oligos (#1 and #3 in Chart 2) have also been synthesized in the phosphorothioated form. We are in the process of transfecting the latter into C3L5 cells.

Although we have repeatedly confirmed the expression of eNOS gene in C3L5 cells by RT-PCR Southern blot analysis, a clear demonstration of e NOS mRNA expression by Northern blot analysis of total RNA from these cells was found to be difficuilt. This difficulty may be due to the instability of the mRNA. The AU(T)-rich motif in the 182 bp3'-UTR sequence (underlined in Chart 1) may have led to mRNA instability. Furthermore, TNF-α has been reported to shorten the half-life of eNOS mRNA (57) to a great extent without affecting the rate of gene transcription. TNF-α destabilises eNOS mRNA in endothelial cells by increasing the binding activity of the cytosolic proteins to the UC-rich region at 3'-UTR (58). We do not know, however, whether C3-L5 cells synthesize TNF-α and if so, whether if destabilises eNOS mRNA by the same way as in endothelial cells. we would like to test it by treating the cells with protein synthesis inhibitor cycloheximide or with TNF-α synthesis inhibitor Pentoxifylline, and then measuring the mRNA levels.

A



B

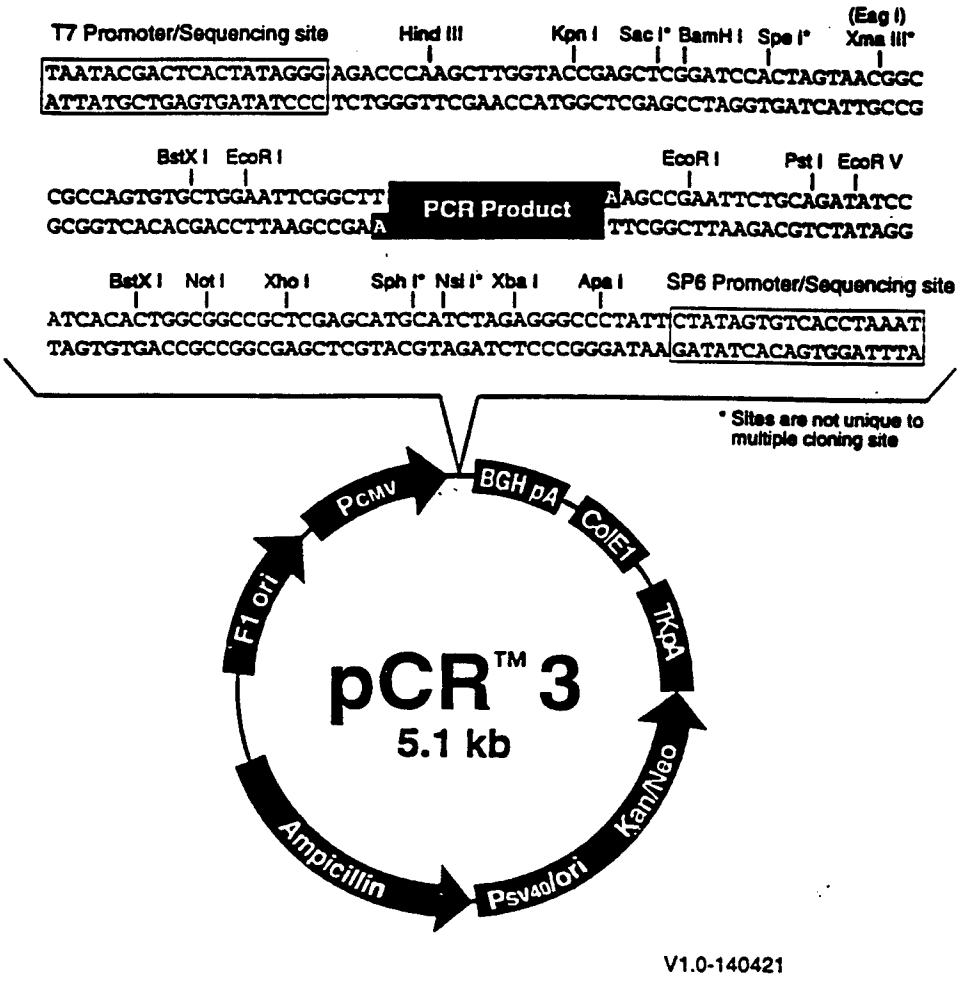
Fig5. Expression of eNOS gene in C3-L5 cell line. RT-PCR analysis of the eNOS in C3-L5 cells. Total RNA (1ug) from lung(positive control) and C3-L5 cells were reverse transcribed and amplified with "one-step supercript" RT-PCR system.

A. Equal amount of RT-PCR reaction products were fractionated by 2% Argarose gel electrophoresiscontaining 0.2mg/ml ethidium bromide, 630bp amplified band visible under UV light observation.

B. Southern-blot hybridization of PT-PCR products with 32P-labled eNOS cDNA probe.

The pCR™3 Vector

Figure of pCR™3 The figure below shows the map of the linearized vector (pCR™3) supplied with this kit.



CMV promotor: bases 1-596
Putative Transcriptional Start: bases 620-625
T7 promotor: bases 638-657
Multiple Cloning Site: bases 664-769
SP6 promotor: bases 774-791
BGH poly A: bases 796-1024
ColE1 origin: bases 1155-1738
TK poly A signal: bases 1923-2194
Kanamycin/Neomycin resistance: bases 2195-3191
SV40 promotor/origin: bases 3192-3549
Ampicillin Resistance: bases 3568-4599
F1 origin: bases 4600-5056

Fig 6. Structure of pCR-3 for antisense cDNA study of eNOS gene in C3-L5 cells

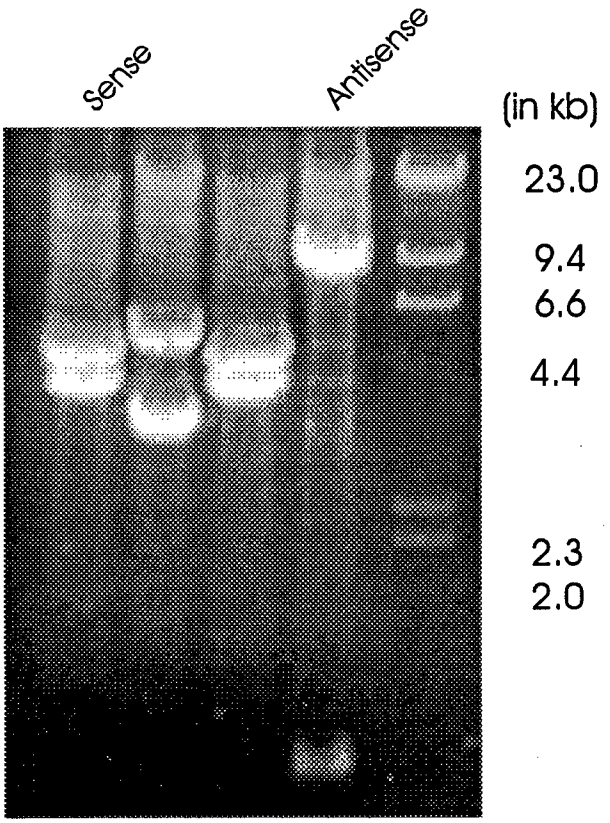


Fig7. Identification of represetitive plasmid subclones of full-length human eNOS cDNA(4.0kb) in pCR-3(5.1kb) vector. Plasmid with sense orientation showed bands at 3.45kb+5.65kb, antisense orientation at 0.7kb+8.5kb.

Fig 8. RT-PCR Southern blot hybridization of some eNOS sense and antisense RNA transfected C3-L5 cells.

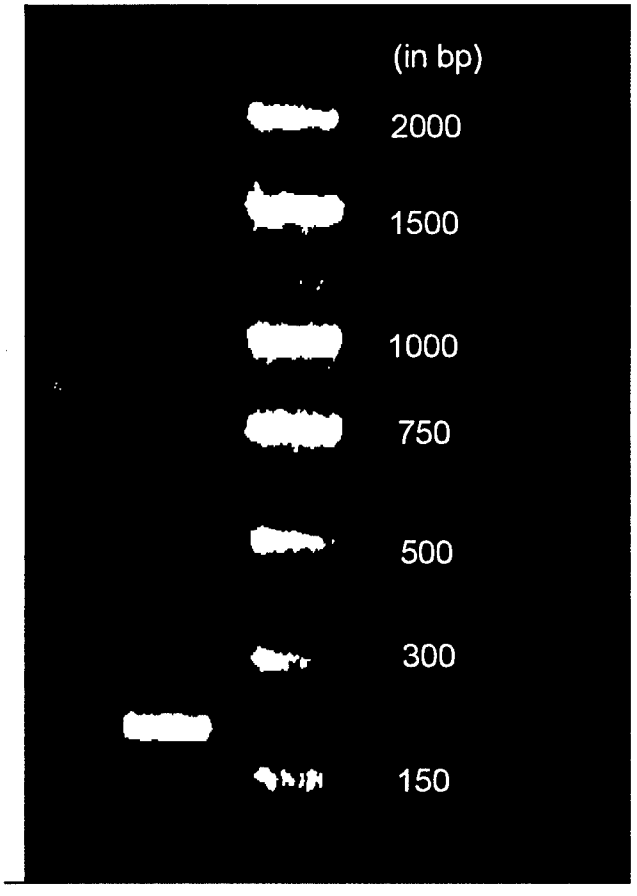


Fig 9. 3'-UTR of murine eNOS cDNA amplified by RT-PCR, 2% Argarose gel electrophoresis under UV light observation

Lane 1. amplified band from mouse lung total RNA.

Lane 2. PCR marker.

Chart 1

cDNA sequence of 3'-UTR of murine Endothelial Nitric Oxide Synthase
Total RNA from adult lungs

5'-TACCTGGTTCCTGACAGTCT→ TTGGAATTCATTTCTCTGTTTTGTGTCAGATAAC
TTCAAAGTACCTTGGCTGTTGCAAAACTTCTTTGTTTTATTTTTAGCTTAATATATAA
GTCTGATGCTCACTTTGACAAATTACATTCAGATACAACCCTCCCTTGTG←CTCAC
ATCTGTCCAGAGGCT-3'

SOURCE: 1-182 bp

STOP CODON: 12-14 bp

Poly A signal and poly A site were not found (or included)

cDNA sequence of 3'-UTR of murine Endothelial Nitric Oxide Synthase
Total RNA from fetal lungs

5'-TACCTGGTTCCTGACAGTCT→ TTGGAATTCATTTCTCTGTTTTGTGTCAGATAAC
TTCAAAGTACCTTGGCTGTTGCAAAACTTCTTTGTTTTATTTTTAGCTTAATATATAA
GTCTGATGCTCACTTTGACAAATTACATTCAGATACAACCCTCCCTTGTG←CTCAC
ATCTGTCCAGAGGCT-3'

SOURCE: 1-182 bp

STOP CODON: 12-14 bp

Poly A signal and poly A site were not found (or included)

Chart 2

Antisense Oligonucleotides Against Murine Endothelial Nitric Oxide Synthase

1) 5' end including start coden (24 Mer):

Antisense	5'-ACACTCTTCAAGTTGCCCATGCCA-3'
Sense	5'-TGGCATGGGCAACTTGAAGAGTGT-3'
Mismatched	5'-AGACACATCTAGTAGCCGTTGGCA-3'

2) 3' end including stop coden (23 Mer):

Antisense	5'-CAAAGACTGTCAGGAACCAGGTA-3'
Sense	5'-TACCTGGTTCCTGACAGTCTTTG-3'
Mismatched	5'-GAATGAGTGACACGAAGCACGAA-3'

3) 3'-UTR #1 (21 Mer):

Antisense	5'-GACACAAAACAGAGAAATGAA-3'
Sense	5'-TTCATTTCTCTGTTTTGTGTC-3'
Mismatched	5'- GTCTGATAAGAGTGATATGAT-3'

4) 3'-UTR #2 (22 Mer):

Antisense	5'-GGGAGGGTTGTATCTGAATGTA-3'
Sense	5'-TACATTCAGATACAACCCTCCC-3'
Mismatched	5'- CGGACGCTTCTAACTCAAAGTT-3'

5) 3'-UTR #3 (19 Mer):

Antisense	5'-AGCCTCTGGACAGATGTGA-3'
Sense	5'-TCACATCTGTCCAGAGGCT-3'
Mismatched	5'-TGCGTCTCGTCAGTTGAGT-3'

6) Coding region against calmodulin site but eNOS unique cDNA sequence (18 Mer):

Antisense	5'-TCTTCACTGCATTGGCTA-3'
Sense	5'-TAGCCAATGCAGTGAAGA-3'
Mismatched	5'-CATCTTCTCTCCTTTCGC-3'

7) Coding region against HADPH-A site but eNOS unique cDNA sequence (25 Mer):

Antisense	5'-AGTGACATCGCCGCAGACAAACATA-3'
Sense	5'-TATGTTTGTCTGCGGCGATGTCACT-3'
Mismatched	5'-TGTCACTTCCCCGGAGTCTAAGATT-3'

Task 2 **Identification of mechanisms of tumor progression which are stimulated by NO.** We hypothesized that tumor-derived NO facilitates tumor progression and metastasis by **(a)** promoting tumor cell invasive ability, **(b)** promoting tumor cell migratory ability and **(c)** promoting tumor-induced angiogenesis which is critical for the growth of solid tumors. We had already shown that tumor-derived NO exerted no influence on tumor cell proliferation *in vitro*. Others have shown that tumor-derived NO promotes tumor blood flow and microcirculation which can indirectly promote tumor growth (59-61).

(a) **Effects of tumor-derived NO on the invasiveness of C3L5 tumor cells.** The results up to date have been detailed in the submitted manuscript in Appendix 4. Some of the data was presented at the "Era of Hope" meting (Appendix 5). Here we provide a comprehensive summary.

Materials and Methods:

C3L5 tumor cells were tested for their invasive ability in an *in vitro* invasion assay (62) designed in our laboratory. In brief, invasive ability of tumor cells were measured from the ability of the cells to transgress a reconstituted basement membrane (matrigel) barrier.

To transgress this barrier, tumor cells have to degrade the basement membrane components and then migrate through the degraded matrix. Tumor cells were prelabeled with 3HThymidine (3HTdR) for a 48 hr period in culture, washed, and placed on a matrigel-coated millipore membrane (8 μm pore size) which forms the bottom of a transwell. The transwells are then placed in tissue culture wells to create an upper and a lower chamber inclusive of tissue culture medium. The degree of invasion is computed as the percent of radioactivity (β counts) appearing in the bottom chamber and the undersurface of the transwell during the assay period (24-72 hrs). The effects of adding NOS inhibitors (NMMA, L-NAME in various concentrations) with or without excess L-arginine (which competes with NOS inhibitors and abolishes their effects), or adding NOS inducers (LPS + IFN-γ) with or without NOS inhibitors were investigated. Simultaneously, iNOS expressioin was analyzed by Northern analysis and the level of NO production was measured in the medium. The latter was done by measuring NO + NO_3 levels by colorimetry following reaction with Griess-reagent.

Results (detailed in Appendix 4):

(a) Presence of either NOS inhibitor (NMMA or L-NAME) reduced the invasion index. This reduction was associated with a parallel reduction in the level of NO produced (Figures 4 and 5, Appendix 4).

(b) Additional presence of excess Larginine in the assay along with NOS inhibitors abrogated the anti-invasive effects of NOS inhibitors (Figure 4, Appendix 4).

(c) Addition of LPS + IFN-γ led to strong induction of iNOS mRNA (Figure 3A, Appendix 4) stimulation of NO production (Figure 3b, appendix 4) and a simultaneous stimulation of invasiveness (Figure 6, appendix 4).

(d) Inclusion of NOS inhibitors with LPS + IFN-γ caused a partial abrogation of the stimulation of NO production and invasiveness by LPS + IFN-γ (Figures 3b and 6, Appendix 4). It was likely that the NO induction was too high for the inhibitors.

Conclusions:

These results show that tumor-derived NO under native conditions as well as conditions of stimulation by LPS + IFN-γ promoted the invasiveness of C3L5 cells. This was abrogated with NOS inhibitors under native conditions, but this abrogation was partial under inductive conditions.

(b) Mechanisms underlying the invasion promoting effects of NO.

Invasion is a multistep process requiring attachment of cells to the constituents of the basement membrane or extracellular matrix (ECM) via integrins or non-integrin receptors, degradation of the ECM components by secretion of a variety of matrix degrading enzymes, most typically, matrix metalloproteases (MMP's) and migration of cells through the degraded matrix. Since matrix degradation is the most critical step of all, we examined whether NO-mediated stimulation of invasiveness of C3L5 cells was due to an upregulation of MMP's or a downregulation of natural MMP inhibitors - tissue inhibitors of metalloproteases (TIMP's), that is, an alteration in the balance between MMP's and TIMP's.

Method

Northern analysis was carried out with total RNA extracted from C3L5 cells grown under different experimental conditions using probes for MMP-2 (72 Kda Type IV collagenase or Gelatinase A), MMP-9 (92 Kda Type IV collagenase or Gelatinase B), TIMP-1, TIMP-2 and TIMP-3. Phosphoimage analaysis of Norther blots relative to 18 S ribosomal RNA (loading controls) provided a measure of mRNA expression under different experimental conditions such as: control untreated cultures, cells cultured with IFN-γ and LPS (to induce iNOS which has been shown to increase NO production by these cells) with or without NOS inhibitor NMMA, and cells cultured with NMMA alone (to block endogenous NO production via eNOS).

Results and Conclusions (detailed in Appendix 4):

(i) C3L5 cells expressed eNOS but not iNOS under native conditions, however, iNOS was induced in the presence of IFN-γ and LPS (Figure 3A, Appendix 4).

(ii) NMMA treatment alone (to block eNOS, a constitutive and low level NO generator), did not alter MMP-2 (72K Da collagenase) expression but upregulated the expression of TIMP-2 and to a smaller level TIMP-3. This indicated that invasion stimulating effects of endogenous NO are, at least in part, mediated by downregulation TIMP-2 and possibly TIMP-3 (Figure 7, Appendix 4).

(iii) IFN-γ and LPS treatment upregulated MMP-2 and downregulated TIMP-3. This indicated that higher levels of induced NO stimulated MMP-2 production in addition to suppressing TIMP-3 production (Figure 7, Appendix 4).

(iv) Addition of NMMA to IFN-γ + LPS treatment restrained MMP-2 expression to control levels, however, only partially restored TIMP-3 expression. This explained the incomplete abrogation of IFN-γ + LPS-induced NO production and invasiveness when cells were treated with NOS inhibitors (Figure 7, appendix 4).

In conclusion, invasion promotion by NO in this tumor system was explained by an alteration in the balance between MMP-2 and TIMP-2/TIMP-3. An upregulation of the former occurred at high NO levels, and a downregulation of the latter occurred at all NO levels. Further studies are needed to test whether the serine protease plasminogen activator (PA) is produced by C3L5 cells and whether it is influenced by NO production; and whether its inhibitors PAI-1 and PAI-2 are also affected. This is because MMP-2 is activated by PA. Indeed, NO has been shown to upregulate urokinase type PA (uPA) in endothelial cells (63).

(c) A comparison of the *in vitro* invasiveness of C3L5 and C10 tumor cells: role of NO.

Materials and Methods:

To examine and compare the invasive ability of these cells and the effect of the NOS inhibitor L-NAME on invasion, an *in vitro* invasion assay was utilized. This assay was essentially similar to the one described in the previous section except for the fact that the assay was non-radioactive, so that visual counts of cells transgressing the matrigel barrier, rather than radioactivity counts were employed. C3L5 and C10 cells were plated at a density of 2.5×10^4 cells/well in cell culture plates on transwells coated with growth factor reduced-Matrigel with either complete RPMI 1640 media, media containing L-NAME (1.0 mM), or media

containing both L-NAME (1.0 mM) and L-arginine (5.0 mM) which competes with NOS inhibitors. The plates were incubated at 37°C in a humidified CO_2 incubator for 24, 48 or 72 hours. After the specified period of time, the transwell membranes were processed utilizing the Diff-Quik® Stain Set. This involved removing and discarding the cells from the upper surface of the membrane (cells which did not invade through the basement membrane), and fixing and staining the cells that had invaded through the basement membrane and appeared on the lower surface of the membrane. Invasive ability was then determined by counting the number of stained cells on the membrane in 5 non-overlapping fields at 400x magification under the microscope.

Results and Conclusions:

The cumulative number of both C3L5 and C10 cells iinvading matrigel increased over time (24 to 72 hours after initial incubation) indicating their normal invasiveness. The invasiveness of C10 cells was significantly different from (lower than) C3L5 cells only at 72 hours. The invasiveness of both cell lines was inhibited in the presence of the NOS inhibitor L-NAME (Figure 10). This inhibition was abrogated in the presence of excess L-arginine in both cases. Thus both cell lines exhibited endogenous NO mediated promotion of invasiveness, which were not clearly distinguishable from each other in the present assay. These experiments are currently being repeated.

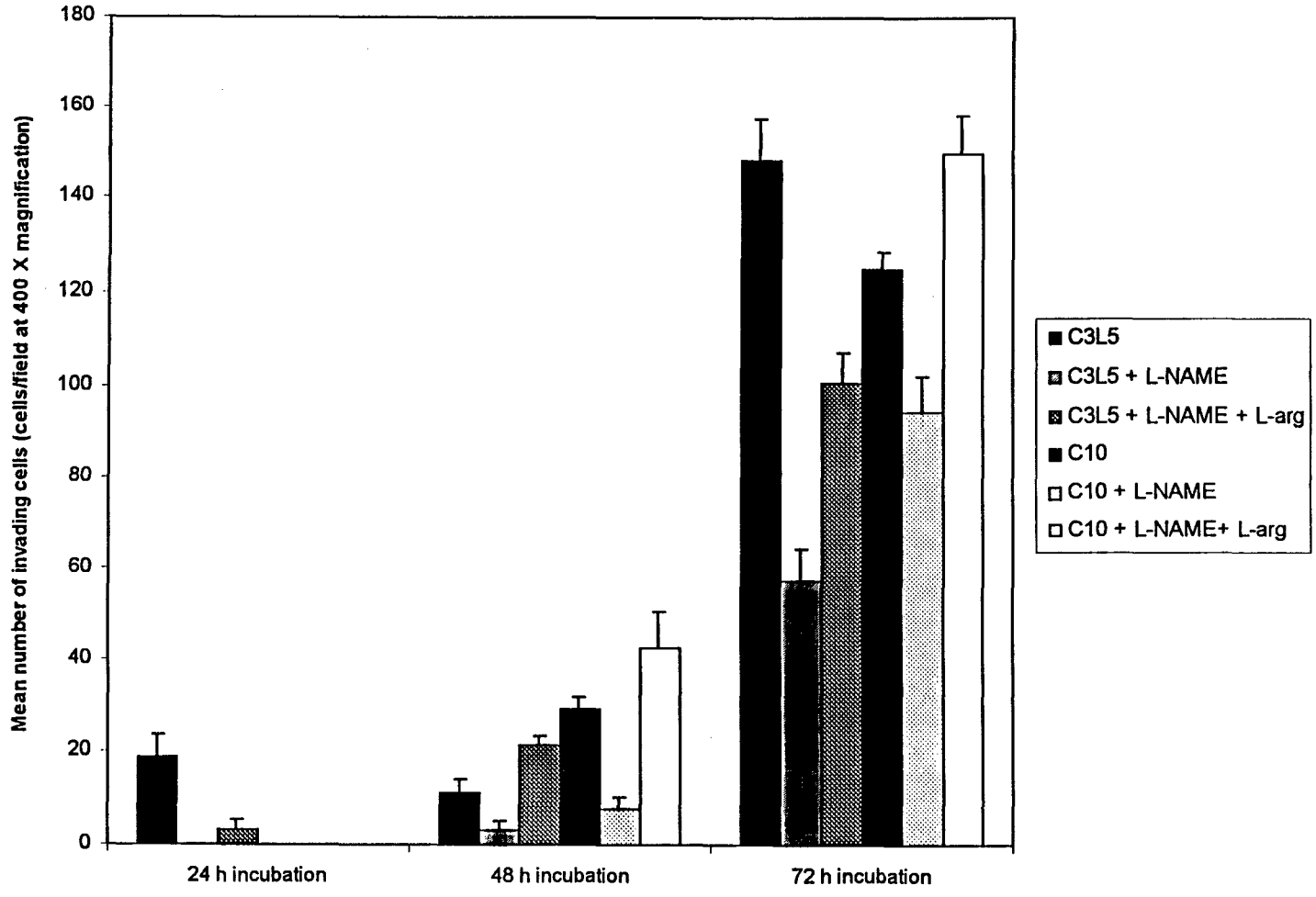


Figure 10. Matrigel invasion by C3L5 and C10 cells treated with L-NAME (1 mM) with or without competition by L-arginine (5 mM) at 24, 48 and 72 h after initial incubation. At 48 and 72 h, L-NAME significantly reduced the number of C3L5 and C10 cells which were able to invade the Matrigel. Invasive ability increased (sometimes to above basal levels) after competition of L-NAME with L-arginine.

(d) Effects of tumor-derived NO on tumor-induced angiogenesis.

Our very preliminary results were summarized in Appendix 1. Currently, we have obtained substantial data in this area, some of which were presented at the last meeting of the American Association for Cancer Research (Appendix 7).

Materials and Methods:

We devised an angiogenesis assay adapted from Kibbey et al. (64). Rehydrated matrigel (reconstituted basement membrane) which is liquid at 4°C and solidified at body temperature, was implanted subcutaneously in mice. The matrigel pellet, when using conventional matrigel, stimulates ingrowth of new blood vessels from the periphery of the implant possibly because of presence of angiogenic factors in the conventional matrigel. We used growth factor-reduced matrigel (obtainied from collaborative research) and found that it stimulated little or no angiogenesis on its own, but stimulated strong angiogenesis when we included C3L5 tumor cells in the matrigel, and recovered the pellet at 7-14 days later. In pilot experiments, we have varied the matrigel concentration, the matrigel volume and the tumor cell numbers to obtain optimal conditions for measurble angiogenesis (gross examination of the pellet and score of number of vessels in histological sections) in the implants placed in C3H/HeJ mice. The following was the protocol for definitive experiments.

Thirty female C3H/HeJ mice (6-8 weeks old) were subcutaneously injected with C3L5 mammary adenocarcinoma cells suspended in Growth Factor Reduced Matrigel® (5×10^4 cells in 3.5 mg Matrigel in 0.5 ml minimum essential essential medium), and on the contralateral side as controls, Matrigel alone (3.5 mg Matrigel in 0.5 ml minimum essential medium). The role of NO in the angiogenic response was evaluated by administering the NOS inhibitor N^G-Nitro-L-arginine Methyl Ester (L-NAME) or its inactive enantiomer N^G-Nitro-D-arginine Methyl Ester (D-NAME) using osmotic minipumps (Alzet Corporation), which provided a constant systemic supply of drug for the duration of the experiment (14 days). Upon sacrifice, implants were removed and divided in half, therefore paraffin and frozen sections could be obtained from the same implant. Samples fixed in 4% paraformaldehyde, processed for paraffin embedding and sectioned, were stained with Masson's Trichrome. Alternatively sections frozen in O.C.T. were sectioned and analyzed immunohistochemically for CD31 (PECAM), an endothelium specific antigen. Both types of sections (i.e., Masson's Trichrome stained or CD31 immunostained) were scanned at low power for areas of highest neovascularization (researcher blind to experimental condition); areas of maximum blood vessel density (i.e., 'hot spots') were imaged at 160X magnification and individual counts obtained.

Immunohistochemical Staining for CD31 Antigen (PECAM-1)

Frozen sections were fixed in ice-cold methanol; endogenous peroxidase activity was blocked in methanol containing 3% H_2O_2 prior to application of blocking antibody: normal mouse serum (1:10; 1 hour at room temperature). Sections were then incubated with primary antibody: purified rat anti-mouse CD31 monoclonal antibody (1:50; overnight at 4°C) followed by secondary antibody; biotinylated mouse anti-rat IgG-2a monoclonal antibody (1:100; 1 hour at room temperature). Negative controls were incubated with rat IgG-2a antibody in place of primary antibody.

Data Analysis

Data were analyzed using the SAS v6.12 program on a Unix mainframe, and treatment groups (i.e., L-NAME and D-NAME) compared using one way analysis of variance (ANOVA). For each treatment group (n=15 mice per group) results were expressed as 1) the mean of the maximum number of microvessels in a single field (160X magnification), and 2) the mean number of microvessels in 3 fields of maximum blood vessel density (160X magnification). A probability of 0.05 was used in determining statistical significance.

Results

Gross Morphology of Implants

Figure 11 shows the gross morphology of tumor-exclusive implants (Figure 11A), and tumor-inclusive implants obtained from L-NAME and D-NAME-treated (Figures 11B and 11C, respectively) animals. Tumor-exclusive implants are small, translucent and avascular. Tumor-inclusive implants are larger, and implants obtained from L-NAME-treated animals are smaller and less vascular than those obtained from D-NAME-treated animals.

Histological Evaluation of Vascularity of Implants—Masson's Trichrome Staining

Figure 12 shows Masson's Trichrome staining of tumor-exclusive Matrigel implant (Figure 12A), and tumor-inclusive implants obtained from L-NAME (Figures 12B and 12C) and D-NAME-treated animals (Figures 12D and 12E). This method stains fibrous tissue stroma bluish-green. Blood vessels containing red blood cells stand out because of bright red staining of red blood cells. Other cells (including tumor cells) show pink staining of cytoplasm and dark magenta coloured nuclei. Implants obtained from both treatment groups consist of 1) a peripheral zone of stroma containing blood vessels, 2) healthy tumor areas, and 3) centrally located, necrotic areas heavily infiltrated with leukocytes. Tumor-exclusive Matrigel implants obtained from L-NAME and D-NAME-treated animals are avascular and contain a few fibroblasts. Areas of highest microvascular count tend to be

localized in the stroma of tumor-inclusive implants. The stroma of tumor-inclusive implants obtained from L-NAME-treated animals appears thinner and less vascular relative to those obtained from D-NAME-treated animals.

Histological Evaluation of Vascularity of Implants—CD31 Immunostaining

Figure 13 shows immunohistochemical localization of CD31 antigen in implants obtained from L-NAME and D-NAME-treated animals (Figures 13A and 13B, respectively). This method stains endothelial cells brown, and localizes endothelial cells within the tumor component of the implants; nuclei are lightly counterstained with Mayer's Haemalum. Neovascularization is reduced in tumor-inclusive implants obtained from L-NAME-treated animals relative to those obtained D-NAME-treated animals.

Quantification of Tumor-induced Neovascularization—Masson's Trichrome Staining and CD31 Immunostaining

Figure 14 shows results obtained for the quantification of blood vessels per unit area for Masson's Trichrome stained and CD31 immunostained sections; data is expressed as the maximum (mean ± S.E.; n = 10-15 animals per group) number of blood vessels per field (160X magnification) and as the average number of blood vessels in 3 fields of maximial density (mean ± S.E.; n = 10-15 animals per group) (160X magnification). Quantification revealed that the neovascular response was reduced in L-NAME-treated mice relative to those treated with D-NAME; this response was evident in Masson's Trichrome sections as well as those examined immunohistochemically for CD31 antigen. Tumor-induced neovascularization, measured by Masson's Trichrome, was reduced in implants obtained from L-NAME-treated animals when data was expressed as 1) the maximum number of blood vessels per field (L-NAME, 52.27 ± 5.9; D-NAME, 92.29 ± 11.3, $P < 0.003$) and 2) as the average number of blood vessels in 3 fields (L-NAME, 42.13 ± 5.4; D-NAME 83.24 ± 10.2, $P < 0.001$) (Figures 14A and 14B, respectively). L-NAME treatment reduced tumor-induced neovascularization, as measured by CD31 immunostaining, when data was expressed 1) as the maximum number of blood vessels per field (L-NAME, 50.8 ± 7.6; D-NAME, 79.9 ± 6.5, $P < 0.0099$) and 2) as the average number of blood vessels in 3 fields (L-NAME, 69.64 ± 4.8; D-NAME, 40.13 ± 5.6, $P < 0.0009$) (Figures 14C and 14D, respectively).

Conclusions:

Endogenous NO promoted angiogenesis in implants of C3L5 tumor cells because this was significantly inhibited by continuous parenterel administration of the NOS inhibitor L-NAME in implant-bearing mice.

Further analyses are underway to correlate the degree of tumor growth in these implants by morphometry of tumor and nontumor areas in implant sections.

Legends for Figures 11, 12, and 13

Figure 11. Gross Morphology of Matrigel Implants: Gross morphology of tumor-exclusive implants (Figure 11A), and tumor-inclusive implants from L-NAME and D-NAME-treated (Figures 11B and 11C, respectively) animals.

Figure 12. Histological Evaluation of Vascularity of Implants—Masson's Trichrome Staining: Masson's Trichrome staining of tumor-exclusive Matrigel implant (Figure 12A), and tumor-inclusive implants obtained from L-NAME (Figures 12B and 12C) and D-NAME-treated (Figures 12D and 12E) animals.

Figure 13. Histological Evaluation of Vascularity of Implants—CD31 Immunostaining: Immunohistochemical localization of CD31 antigen in implants obtained from L-NAME and D-NAME-treated (Figures 13A and 13B, respectively) animals.

Figure 11

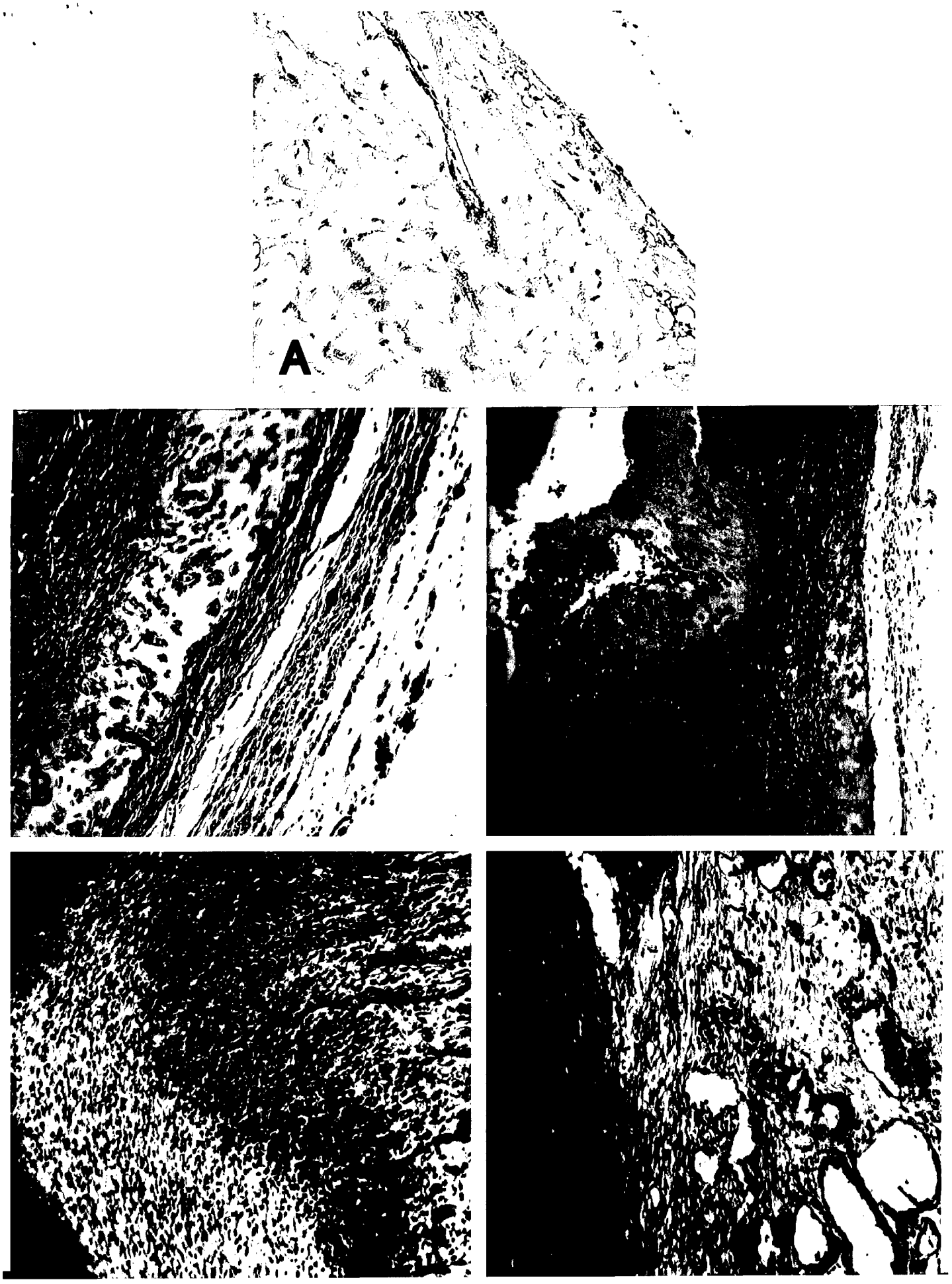


Figure 12

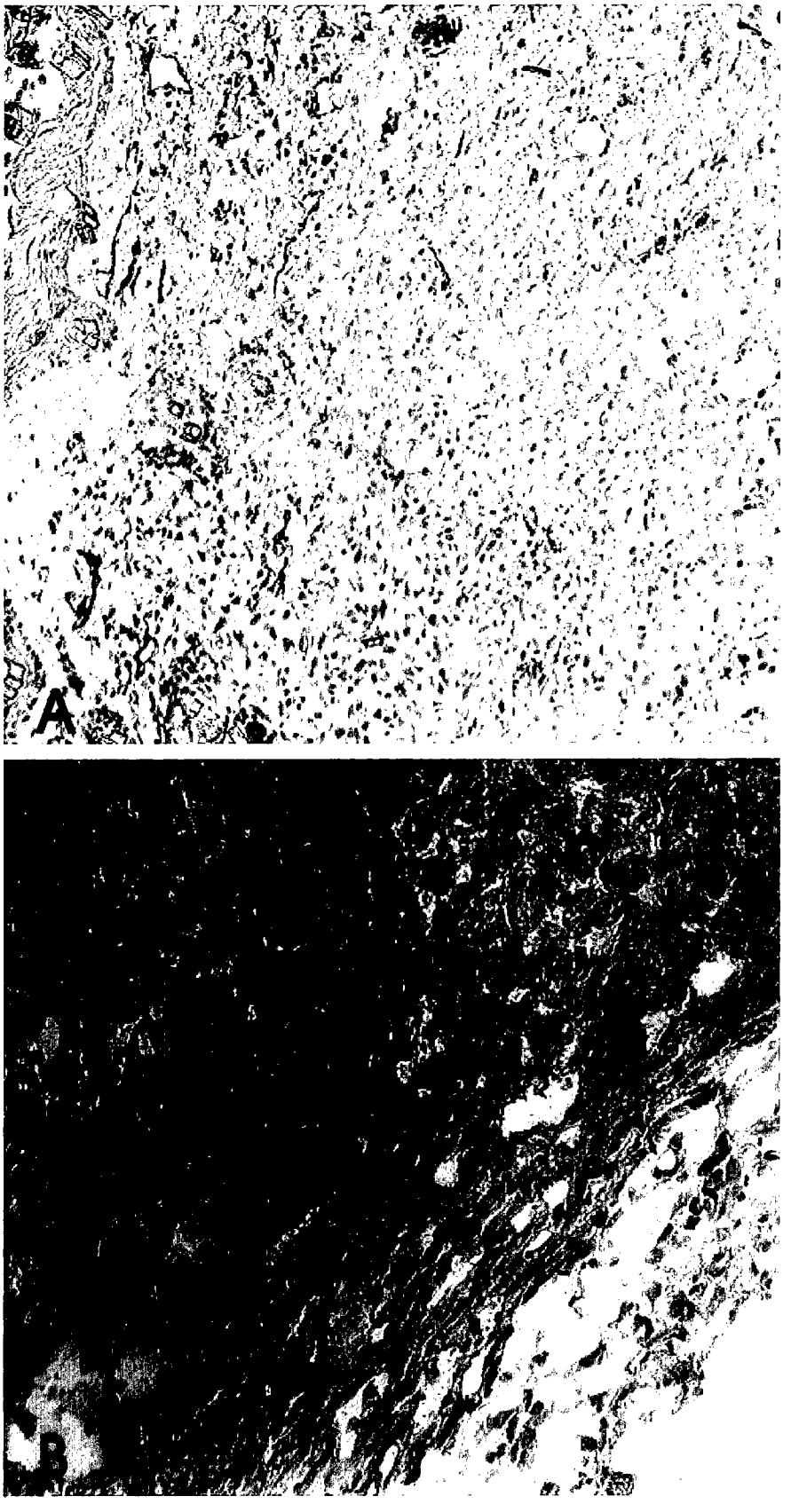


Figure 13

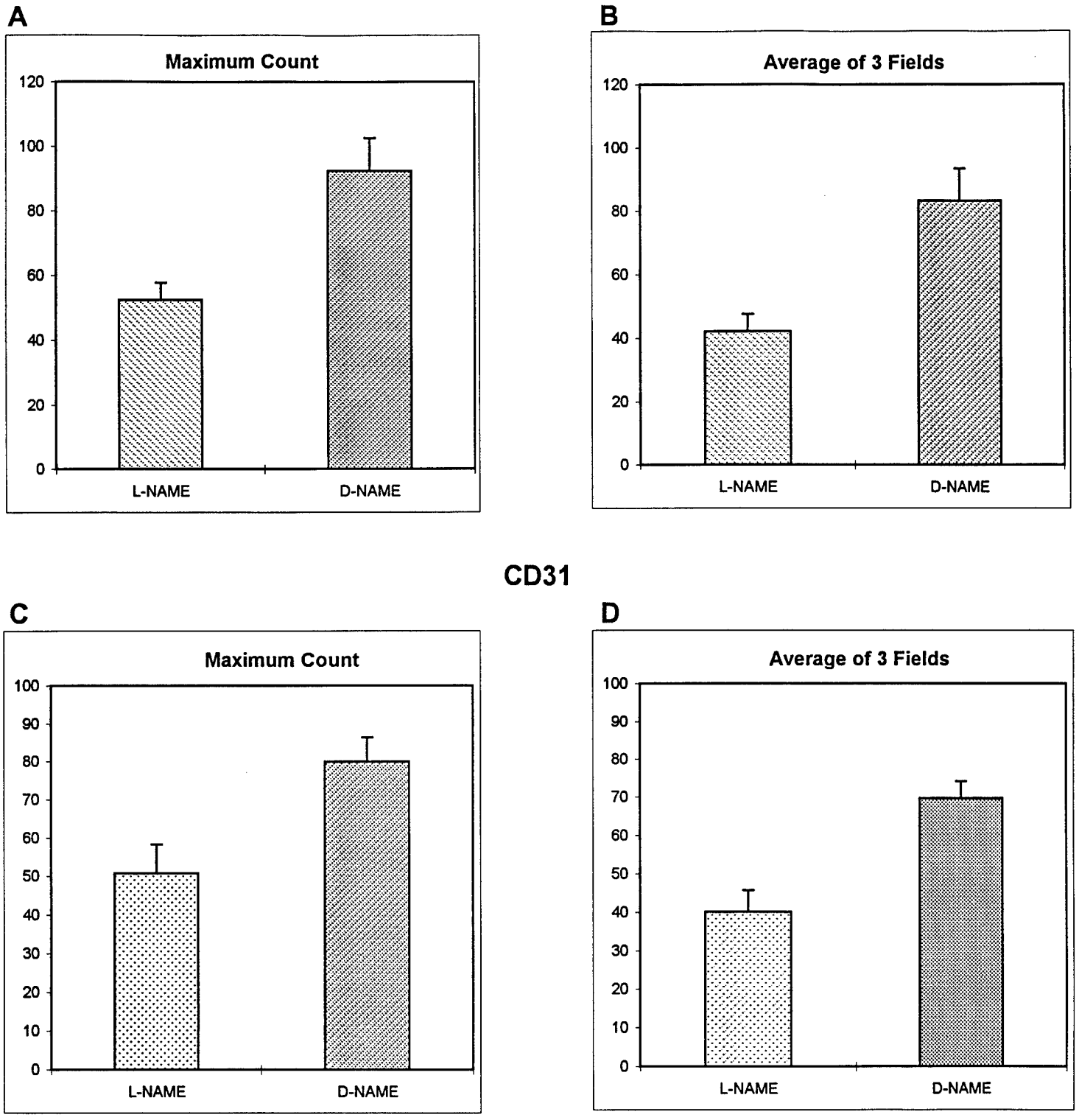


Figure 14. Quantification of Blood Vessels Per Unit Area—Masson's Trichrome Staining and CD31 Immunostaining: Tumor-induced neovascular response, obtained from Masson's Trichrome stained sections (Figures 14A and 14B) and CD31 immunostained sections (Figures 14C and 14D). Data are expressed as the maximum number of blood vessels per field (Figures 14A and 14C) and as the average number of blood vessels in 3 fields (Figures 14B and 14D).

Task 3 **Identification of the cellular source of NO-production responsible for IL-2 induced capillary leakage and mechanisms by which this NO-production compromises antitumor effects of IL-2 therapy.**

This task, as initially proposed, was essnetially completed in Year I, presented in the last year's progress report and published (52). We showed that IL-2 therapy induced active iNOS in tissues contiguous with pleural effusion and caused structural damage to the lungs and its capillaries. These injuries were ameliorated with the NOS inhibitor L-NAME.

These results raised the following questions. Was the damage to the lungs and its capillaries due to a direct injury (structural damage and apoptosis) by NO, or injury by certain reaction product of NO? Recently, it has been reported that oxygen-free-radicals play a role in IL-2 therapy induced capillary damage because it could be ameliorated with dimethylthiourea, a scavanger of oxygen-free-radicals (65). We hypothesise that formation of peroxinitrite, a potent endotheliotoxic molecule, due to a combination of NO with superoxide may be the strongest mediator of IL-2 induced capillary leakage. This hypothesis will be tested by immunostaining for nitrotyrosine in the lungs and lung capillaries in mice suffering from IL-2 induced pulmonary edema. Since cytotoxicity due to peroxinitrite is reported to be due to nitration of tyrosine-residues of intracellular tyrosine-kinases to form nitrotyrosine, nitrotyrosine provides a good marker for peroxinitrite mediated cellular injury: If our hypothesis is correct, this marker should appear in IL-2 treated mice, and disappear or diminish in mice treated with IL-2 in combination with a NOS inhibitor L-NAME. In addition, we should be able to demonstrate cytoxic effects of peroxinitrite on endothelial cells *in vitro*. These studies will be conducted in Years III and IV.

7. OVERALL CONCLUSIONS

Following were the achievements during the project period.

1. We have expanded and validated our earlier data showing that:

(a) Spontaneous primary C3H/HeJ tumors show a heterogeneity in eNOS-bearing tumor cells; this expression was unrelated to tumor growth rate. However, metastatic foci resulting from each primary tumor was mostly eNOS positive.

(b) All C3L5 tumor cells (a highly metastatic clone of a spontaneous tumor) expressed eNOS *in vitro*; a minority expressed iNOS under inductive conditions (IFN-γ + LPS). When transplanted *in vivo,* most tumor cells at the primary site and a high proportion at the metastatic site expressed eNOS. C10 tumor cells originally

shown to be a poorly metastatic clone of the same spontaneous tumor were shown to have a lower *in vivo* growth rate of primary tumors and a lower rate of spontaneous lung metastasis than C3L5 cells. These differences were positively correlated with their differences in eNOS protein expression *in vitro* as well as *in vivo* in primary tumors but not in metastatic foci. Their *in vitro* invasive ability were not, however clearly distinguishable from each other.

These findings substantiated further our hypothesis that eNOS expression provided an advantage for metastasis.

2. We have abandoned our futile attempts to knockout eNOS gene in C3L5 cells because we find that they have increased (3.6) number of gene copies. We have since adopted the alternative approach of downregulating eNOS by antisense transfection. We have made significant progress in isolating eNOS downregulated clones. They will be tested for eNOS activity and if found to have stable suppression of eNOS activity will be applied to functional assays. Simiultaneously, we shall apply antisense oligos in short-term biological assays.

3. **(a)** We have shown that endogenous NO promoted invasiveness of C3L5 and C10 tumor cells. The invasive function of the highly metastatic C3L5 cell line was investigated in detail. Their invasiveness was further stimulated by additional NO production when treated with IFN-γ and LPS because of the induction of iNOS in tumor cells.

This is the first definitive evidence of NO-mediated promotion of tumor cell invasiveness.

(b) We have partially identified the mechanisms responsible for NO-mediated stimulation of invasiveness. Endogenous and IFN-γ + LPS-induced NO downregulated the expression of TIMP-2 and TIMP-3 genes. Induced NO further upregulated the expression of MMP-2 gene. Thus, NO-mediated promotion of invasiveness resulted from an alteration in the balance between MMP-2 and TIMP's.

This is the first demonstration of mechanisms for NO-mediated promotion of tumor cell invasiveness.

4. By devising a novel tumor angiogenesis assay *in vivo,* we have obtained substantial data showing that endogenous NO promotes C3L5 tumor-induced angiogenesis.

5. We had shown that active, inducible NOS expression, leading to high NO production *in vivo* is responsible for IL-2 therapy-induced capillary leakage in healthy mice. We identified the iNOS-expressing cells in the vicinity of the leakage

(pulmonary edema, pleural effusion) and have shown that NOS inhibitors can restrain the IL-2 therapy-induced structural damage to the lungs. We shall conduct further studies to test our hypothesis that NO-mediated capillary damage following IL-2 therapy is owing to the formation of peroxinitrite.

In summary, our progress matched with our expectations in some areas. In other areas we had an accelerated progress, leading to some newer proposals for experimentation within the overall objectives of the project.

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APPENDICES

1. Lala PK and Orucevic A: Role of nitric oxide in tumor progression: Lessons from experimental tumors. Cancer and Metastasis Reviews. 17: 91-106, 1998.

2. Orucevic A and Lala PK: Role of nitric oxide in interleukin-2 therapy induced capillary leak syndrome. Cancer and Metastasis Reviews. 17: 127-142, 1998.

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   This is an overview, which I was invited to write as the guest editor of Cancer and Metastasis Reviews, Vol. 17, entitled "Nitric Oxide and Tumor Progression".

   Since this article contains no original data, I have not acknowledged any granting agency in this article. It is appended to give the reviewers a feel of the field and the topics covered in this issue of the journal.

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Role of nitric oxide in tumor progression: Lessons from experimental tumors

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Key words: nitric oxide, invasion, metastasis, angiogenesis, mammary tumor

Abstract

Nitric oxide (NO), a potent biological mediator, plays a key role in physiological as well as pathological processes, including inflammation and cancer. The role of NO in tumor biology remains incompletely understood. While a few reports indicate that the presence of NO in tumor cells or their microenvironment is detrimental to tumor cell survival and consequently their metastatic ability, a large body of clinical and experimental data suggest a promoting role of NO in tumor progression and metastasis. We suggest that tumor cells capable of very high levels of NO production die *in vivo,* and those producing or exposed to lower levels of NO, or capable of resisting NO-mediated injury undergo a clonal selection because of their survival advantage; they also utilize certain NO-mediated mechanisms for promotion of growth, invasion and metastasis. The possible mechanism(s) are: (a) a stimulatory effect on tumor cell invasiveness, (b) a promotion of tumor angiogenesis and blood flow in the tumor neovasculature, and (c) a suppression of host anti-tumor defense. In this review, we discuss these mechanisms on the basis of data derived from experimental models, in particular, a mouse mammary tumor model in which the expression of eNOS by tumor cells is positively correlated with invasive and metastatic abilities. Tumor-derived NO was shown to promote tumor cell invasiveness and angiogenesis. The invasion-stimulating effects of NO were due to an upregulation of matrix metalloproteases and a downregulation of their natural inhibitors. Treatment of tumor-bearing mice with NO-blocking agents reduced the growth and vascularity of primary tumors and their spontaneous metastases. We propose that selected NO-blocking drugs may be useful in treating certain human cancers either as single agents or as a part of combination therapies.

I. Introduction

Progression of solid tumors is a multistage process involving genetic changes in tumor cells that provide selective advantages for growth, invasion, and metastasis due to tumor-derived (autocrine) or host-derived (paracrine) signals capable of promoting these events. Growth of primary as well as metastatic tumors can be facilitated by direct proliferation-stimulating events such as a perpetuation of positive growth-regulating signals, e.g., activation of certain protooncogenes which serve as receptors for proliferation-stimulating growth factors, or production of proliferation-stimulating growth factors. This can also result from a loss of negative growth-regulating signals, e.g., inactivation of certain tumor suppressor genes involved in cell cycle control or receptors for proliferation-blocking growth factors [1, 2]. Tumor growth can also be facilitated indirectly by promotion of tumor angiogenesis and tumor blood flow [3].

For tumor cells to invade into surrounding normal tissues or metastasize to a distant site, a number of steps must be completed successfully [4]. First, tumor cells must bind to one or more constituents of the basement membrane or extracellular matrix

(ECM) via cell surface integrins or non-integrin receptors. This binding is more than an adhesive event; it can also lead to transduction of signals that may facilitate invasion [5]. Second, tumor cells must degrade basement membrane and ECM constituents; this step is facilitated by the production of active matrix degrading enzymes in excess of natural inhibitors of these enzymes [4, 6]. Third, tumor cells must migrate through the degraded ECM. This step is facilitated by the anchoring of cells to the ECM by appropriate integrin(s) [7], and by migration-promoting cell-ligand interactions [8]. Finally, for metastasis to occur, tumor cells must intravasate and survive within the blood vessels or lymphatics, and then extravasate and seed at distant locations. Recent work utilizing live videomicroscopy has demonstrated that even after successful extravasation and seeding, many tumor cells may either die or remain quiescent for a significant period [9]. Thus, successful metastasis often requires additional autocrine/paracrine growth or angiogenesis-stimulating signals at the new site. Similar steps when successfully repeated, may allow metastatic tumors to remetastasize to newer sites.

In the present article we shall discusss the contributory role(s) of nitric oxide (NO) in tumor progression and metastasis in the context of the above events which can influence growth, invasion or metastasis in experimental tumor models.

During the last decade, following the discovery [10] that NO accounts for the full biological activity of endothelium-derived relaxing factor (EDRF) [11], NO has been shown to be produced by many mammalian cells and responsible for numerous physiological functions. These include vasodilation, inhibition of platelet aggregation, modulation of neurotransmission, and mediation of injury by macrophages to bacteria, parasites and tumor cells [12–17]. On the other hand, sustained high levels of NO production in the body can also lead to pathological injuries mediated by NO or its metabolites [18]. NO production depends on conversion of the amino acid L-arginine to L-citrulline by a family of enzymes named NO synthases (NOS) [19–20]. Three isoforms have been identified so far. The endothelial type NOS (eNOS) is a constitutive, Ca^{++} and calmodulin-dependent form of the enzyme, expressed by many cells including endothelial cells, myocardial cells and pyramidal cells of the hippocampus. The neuronal type NOS (nNOS) is also constitutive, Ca^{++} and calmodulin-dependent, and is expressed by certain cells including neurons of the central nervous system, the myenteric plexus, skeletal muscle cells, renal, bronchial and pancreatic islet cells. Inducible type NOS (iNOS) is Ca^{++} and calmodulin-independent, and is expressed by macrophages in many mammalian species, endothelial cells, hepatocytes, cardiac myocytes, chondrocytes and many other cells following stimulation by inflammatory cytokines and/or bacterial endotoxin [20–23]. The expression of iNOS is high in activated rodent macrophages and endothelial cells, and poor in human macrophages [24].

NO is often an important component of the chemical microenvironment of tumors, produced either by tumor cells, endothelial cells in the tumor microvasculature or macrophages and stromal cells within the tumors. Because of its lipophilic nature, NO can rapidly cross cell membranes and enter intracellular compartments to exert its action, even when produced by a neighbouring cell. Thus, it can mediate interactions between tumor cells and host cells. The functional role of NO in tumor biology is complex and remains to be fully defined. While a small number of reports indicate that the presence of NO in tumor cells or in their microenvironment is detrimental to tumor cell survival and consequently their metastatic ability, numerous clinical and experimental studies suggest a promoting role of NO in tumor progression and metastasis. In this review, we discuss these two apparently conflicting views and suggest that the opposing effects of NO may depend on two important variables: the levels of NO production and the genetic makeup of tumor cells. We suggest that in a heterogeneous population of tumor cells, clonal evolution favors those capable of resisting NO-mediated injury. In addition, many tumor cells may also utilize NO in their microenvironment, facilitating some of the steps required for tumor growth, invasion and metastasis; this will be illustrated from our own studies using spontaneous C3H/HeJ mouse mammary adenocarcinomas and their clonal derivatives as an experimental model for tumor progression and metastasis.

II. Association between NO and tumor growth

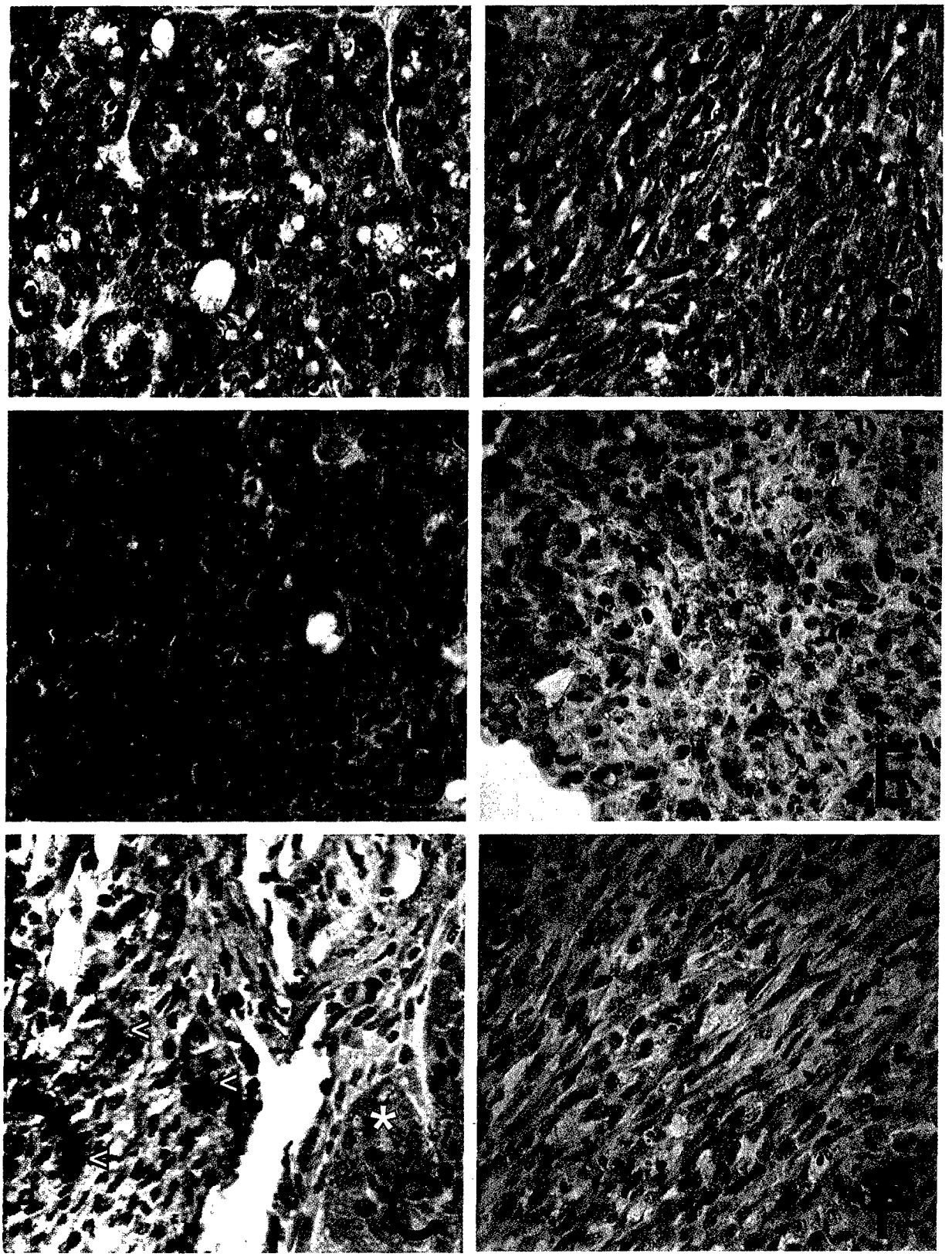
The genotoxic role of NO in promoting carcinogenesis is well recognized. The underlying mechanisms are the subject matter of another article (by Felley-Bosco) in this issue. Chronic exposure of cells to NO can result in multiple genetic changes which may underlie histological changes such as metaplasia and the progression of metaplasia into neoplasia. The presence of eNOS in breast apocrine metaplastic cells of fibrocystic disease in the human [25] and iNOS in macrophages within the hyperplastic stromal tissue of a rat model of Barret's esophagus [26] are believed to promote the progression of metaplastic epithelia into carcinomas.

Human tumor materials, in general, have provided the strongest link between NO production and disease progression. While the functional implications of elevated serum NO levels observed in many cancer patients [27] remain unexplored, a number of reports indicate a contributory role of NO to tumor progression. An abundant expression of NOS, as well as NOS activity, has been positively correlated with the degree of malignancy in human ovarian and uterine cancers [28], central nervous system tumors [29], and breast cancer [30]. Contributing to the elevated NOS activity are constitutive form(s) in tumor cells [28, 29] and/or tumor endothelial cells [29], and the inducible form in the tumor endothelial cells [29] and/or tumor associated macrophages [30].

The relationship of NO to human colonic tumor progression remains controversial. Histochemical localization of NAD(P)H diaphorase enzyme, NOS activity and NOS expression in the human colonic mucosa, polyps and carcinomas suggest an inverse relationship between the enzymes and colonic tumor progression [31, 32]. In contrast, studies of NOS gene expression and NOS activity in a panel of human colonic adenocarcinoma cell lines revealed that all expressed mRNA for the eNOS gene, and some exhibited significant NOS activity [33]. Evidently, more studies are needed in human colonic tumors.

NOS expression has been examined in a number of experimental tumor models. An abundant expression of iNOS by cells of the tumor vasculature has been implicated in the promotion of tumor growth both in murine [34] and rat [35] tumors. eNOS expression by tumor cells is positively correlated with invasiveness and metastasis in a murine mammary adenocarcinoma model, to be described later.

Numerous studies in animal models have provided direct evidence for a stimulatory role of NO in tumor progression. In a rat colonic adenocarcinoma model showing iNOS expression in the tumor vasculature, treatment with N^G-Nitro-L-arginine methyl ester (L-NAME), a potent NOS inhibitor, reduced NO production and tumor growth [35]. Similarly, anti-tumor and anti-metastatic effects of two NOS inhibitors N^G-methyl-L-arginine (NMMA) and L-NAME were observed in our laboratory using a mouse mammary adenocarcinoma model [36, 37], in which tumor cells expressed eNOS. Recently, Edwards *et al.* [38] observed that NO production induced by lipopolysaccharide (LPS) and interferon (IFN)-γ in EMT-6 murine breast cancer cells inhibited cell growth *in vitro*, but stimulated tumorigenesis and experimental lung metastasis *in vivo*. Finally, engineered expression of murine iNOS in a human colonic adenocarcinoma cell line resulting in continuous, moderate levels of NO production *in vitro*, was associated with increased tumor growth and vascularity *in vivo* following transplantation in nude mice [39]. These findings of a facilitatory role of NO in tumor growth and metastasis are in contrast with those reported for murine K1735 melanoma cell lines, in which the level of iNOS expression was inversely correlated with their ability for experimental metastasis [40]. Furthermore, engineered overexpression of iNOS in an iNOS-deficient melanoma line suppressed tumorigenic and metastatic abilities *in vivo* because of NO-mediated cytostasis and apoptosis [41]. Two explanations may be offered for these apparently conflicting results. First, very high NO levels (such as those produced by the iNOS overexpressing murine melanoma line) can be detrimental to tumor cell survival. Indeed, the iNOS overexpressing melanoma line had poor survival in the absence of NOS inhibitors *in vitro* and *in vivo* [41]. Second, tumor cells may vary in their susceptibility to NO-mediated cytostasis and apoptosis, certain tumor cells can not only re-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sist NO-mediated injury, but also utilize NO to facilitate tumor progression and metastasis. These possibilities will be discussed later in more detail.

III. C3H/HeJ spontaneous mammary adenocarcinoma and its clonal derivatives: a model for tumor progression

Approximately 90% of C3H/HeJ female retired breeder mice develop mammary tumors during their lifespan [42]. Despite extensive variation in the site (anywhere in the mammary line) or the time (6 months to 2 years) of tumor appearance, most tumors are highly vascular and exhibit histological features of invasive adenocarcinomas with a pseudoglandular architecture. In general, animals demonstrating spontaneous primary tumors develop eventual metastasis in their lungs. Lung metastasis may occasionally be present even in the absence of a visible or palpable primary tumor [86]. Tumor development in these mice requires the proviral form of the mouse mammary tumor virus (MMTV), which is transmitted via the mother's milk and integrated in the developing mammary tissue of the female offspring. This leads to tumorigenesis owing to insertional mutagenesis of certain important growth-regulating genetic loci which serve as the proviral integration sites [43, 44].

Clonal derivatives of spontaneous mammary tumors exhibited extensive heterogeneity in growth rates observed *in vitro* and *in vivo*, and metastasis formation *in vivo* following subcutaneous transplantation in syngenic mice [45]. Two clones derived from a single spontaneous tumor differed markedly in their abilities for spontaneous lung metastasis from a primary subcutaneous transplant site; C10 was poorly metastatic, and C3 was highly metastatic. However, because the metastatic ability of C3 declined after several years of *in vitro* passage [46], C3 cells were subjected to an *in vivo* selection pressure of 5 cycles of subcutaneous to pulmonary passage, yielding highly metastatic cells [47]. The resulting cell line, designated C3L5, has since maintained a very high ability for spontaneous lung metastasis from subcutaneous sites. The data presented below relate to C3H/HeJ spontaneous mammary tumors as well as the high and low metastatic clonal derivatives, C3L5 and C10, respectively.

IV. Relationship of NO production to tumor growth and metastasis in the C3H/HeJ mammary tumor model

IV.A. NOS expression in spontaneous (primary and metastatic) mammary tumors (Lala, Orucevic and Hum, unpublished)

Random examination of spontaneously developing mammary tumors harvested at 12 weeks of tumor age revealed immunohistochemical evidence of eNOS expression in tumor cells (Figure 1A and B) and iNOS expression in certain macrophages in the tumor stroma (Figure 1C). Endothelial cells in the tumor vasculature were eNOS positive.

In the primary tumors, a heterogenous pattern of eNOS staining was noted; tumor cells in pseudoacinar formation were either strongly positive or negative (Figure 1A). However, the lung metastatic nodules were composed primarily of strongly eNOS positive cells (Figure 1B), suggesting a positive cor-

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Figure 1. Immunostaining patterns for eNOS (A, B, D and E) and iNOS (C) in 12 week old spontaneous (A, B and C) and 3·5 week old transplanted C3L5 (D, E) mammary tumors at the primary (A, C, and D) and metastatic (B, E) sites. Positive immunoreactivity is indicated by brown staining. Blue staining (nuclei, and to a minor extent cytoplasm) is due to counterstaining with hematoxylin.

A Spontaneous primary tumor showing eNOS positive as well as eNOS negative tumor cells arranged in pseudoacinar clusters.

B Lung metastasis of the tumor A showing strong eNOS positivity in most tumor cells.

C Spontaneous primary tumor showing iNOS positive macrophages (<) in the tumor stroma. Tumor cells (*) were iNOS negative.

D Subcutaneous C3L5 primary tumor showing eNOS positivity in nearly every tumor cell.

E Strong eNOS positivity is also seen in the majority of C3L5 tumor cells at the site of spontaneous lung metastasis.

F A negative control for eNOS staining of the primary C3L5 tumor, in which the eNOS antibody was replaced with an Ig of the same isotype.

relation between eNOS expression and metastatic ability.

IV. B. NOS expression in C3L5 and C10 tumor lines (Lala, Orucevic and Hum, unpublished)

C3L5 and C10 tumor lines, when transplanted subcutaneously, both give rise to primary tumors which grow to large sizes. However, C10 tumors grow more slowly, are more circumscribed and less invasive. The mean number of spontaneous lung metastases produced 3 weeks after subcutaneous transplantation of 5×10^5 cells in syngeneic mice (n = 15) was 10.7 (median = 8) with C3L5 cells and 1.3 (median = 1) for C10 cells, respectively.

In vitro cultured tumor lines showed positive eNOS staining in nearly 100% of C3L5 cells (Figure 2A), but weak (and heterogeneous) eNOS staining in C10 cells (data not shown). Both cell lines were negative for iNOS. When cultured in the presence of LPS and IFN-γ, C3L5 cells were induced to express iNOS as shown by strong iNOS staining in 25–30% of cells (Figure 2B). C3L5 cells grown *in vivo* maintained strong positivity for eNOS, both in the primary tumors and their spontaneous lung metastasis (Figure 1C and D). These findings were consistent with the notion that eNOS expression by tumor cells provided a selective advantage for invasion and metastasis in this mammary tumor model.

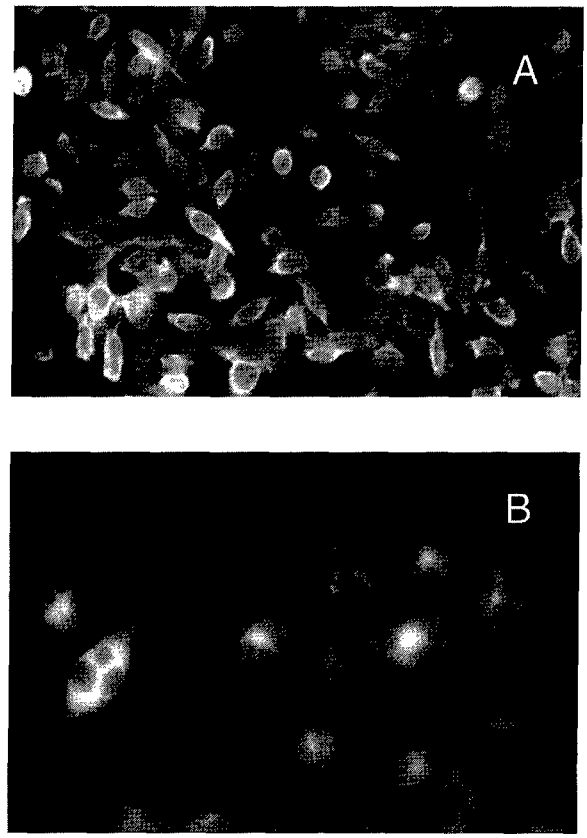

Figure 2. Immunofluorescent staining of cultured C3L5 cells for eNOS (A) under normal culture conditions, and iNOS (B) after 24 h exposure to LPS (10 μg/ml) and IFN-γ (1000 U/ml). Most tumor cells were strongly positive for eNOS (A), and 25–30% of the cells became strongly positive for iNOS (B) following induction with LPS and IFN-γ.

IV.C. Effects of NOS inhibitors (NMMA and L-NAME) on tumor growth and metastasis in C3L5 tumor-bearing mice

Treating C3L5 tumor-bearing mice with two NOS inhibitors [36, 37] provided the first direct evidence of a contributory role of NO in tumor growth and metastasis. NMMA (given repeatedly as two 3 day rounds by the subcutaneous route) as well as L-NAME (administered orally in the drinking water as two 4 day rounds) led to a reduction in the growth of the subcutaneously transplanted primary tumors and their spontaneous lung metastases (Figures 3–6]. The finding of reduced primary tumor growth has since been reproduced with chronic L-NAME therapy administered subcutaneously using osmotic minipumps (Jadeski and Lala, unpublished). Treatment with L-NAME has also been reported to reduce the growth of the primary tumors in a transplanted rat colonic adenocarcinoma model [35], in which the tumor vasculature expressed iNOS. Based on the temporal kinetics of tumor growth after the therapy, the authors suggested that L-NAME reduced blood flow through the tumor vasculature and that native NO was instrumental in promoting the tumor blood flow.

What are the mechanisms underlying NO-mediated promotion of tumor growth and metastasis observed in numerous tumor models? The possibilities, in theory, include: (a) a direct stimulation of tumor cell proliferation; (b) a promotion of tumor

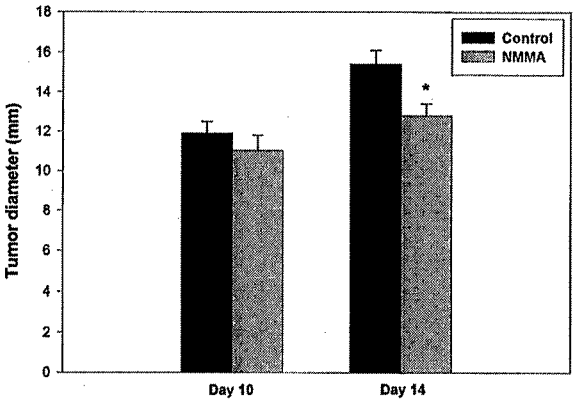

Figure 3. Effects of subcutaneous NMMA therapy for 3 days (20 mg/kg/injection, every 8 hr × 10 injections) on days 10–13 after subcutaneous transplantation of 5×10^5 C3L5 mammary tumor cells, on mean tumor diameter (n = 10–15). There was a significant (* $p < 0.05$) decline in primary tumor size measured on day 14. *(Adapted with kind permission from Orucevic and Lala, Cancer Immunol Immunother, Springer Verlag, 42: 38–46, 1996).*

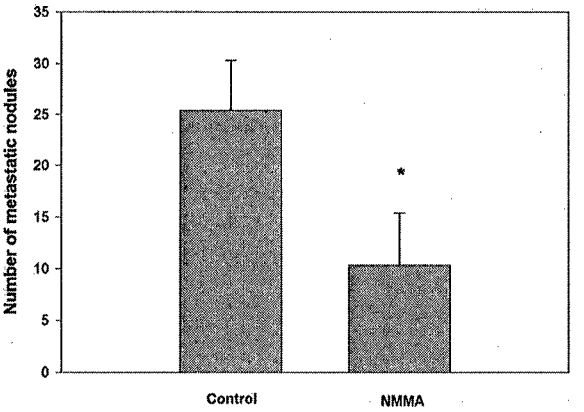

Figure 4. Effects of subcutaneous NMMA therapy given as two 3 day cycles (days 10–13 and 19–22 after subcutaneous C3L5 tumor transplantation, at the same dose rate as in Figure 3), on the number of lung metastatic nodules in mice (n = 9) killed on day 22. The therapy resulted in a significant ($P < 0.05$) reduction in spontaneous lung metastasis. *(Adapted with kind permission from Orucevic and Lala, Cancer Immunol Immunother, Springer Verlag 42: 38–46, 1996).*

cell invasiveness; (c) a promotion of tumor angiogenesis; (d) a promotion of microcirculation in the tumor neovasculature; (e) a suppression of the host anti-tumor defence. Of these, we have gathered evidence in favour of possibilities (b) and (c). The influence of NO in tumor microcirculation is the subject matter of another article (by Fukumura and Jain) in this issue.

V. NO and tumor cell proliferation, survival and apoptosis

Edwards et al. [38] found a discrepancy in tumor cell proliferation *in vitro* and tumor growth *in vivo* after induction of NO with LPS and IFN-γ; *in vitro* induction inhibited tumor cell proliferation, whereas *in vivo* induction promoted tumorigenesis and metastasis. Since C3L5 mammary adenocarcinoma cells expressed active eNOS and produced NO *in vitro*, we tested whether NMMA affected tumor cell proliferation by measuring the uptake of ^{3}H-Thymidine (^{3}HTdR) by C3-L5 cells *in vitro*. NMMA treatment for 24 hours at concentrations capable of blocking NO production *in vitro*, had no influence on ^{3}HTdR uptake when cells were pulsed with ^{3}HTdR during the last 6 hours of 24 hour culture (unpublished data), suggesting that endogenous NO did not directly affect C3L5 tumor cell proliferation.

It has been reported that endogenous or exogenous NO exerts anti-proliferative effects on cells that express functional wild-type p53 tumor suppressor gene [48, 49]. NO stimulates accumulation of the p53 protein in these cells which blocks proliferation by hindering progression of cells through the cell cycle; this may explain cytostatic effects of NO on certain tumor cells: Tumor cells transfected with iNOS were growth-inhibited *in vivo* only when they expressed wild type p53. However, iNOS-expressing tumor cells in which p53 was lost or mutated were resistant to the anti-proliferative effects of endogenous NO, and grew faster *in vivo* than those not expressing iNOS [49].

Apoptosis is another mechanism which can compromise cell survival in the presence of high NO levels. It has been suggested that the NO-dependent component of tumoricidal function of certain immune effector cells is by induction of tumor cell apoptosis [50]. A similar tumoricidal function has also been ascribed to cytokine-activated endothelial cells [51]. Very high levels of endogenous NO production can trigger apoptosis in the NO-producing cell, e.g. in cytokine-activated transformed mu-

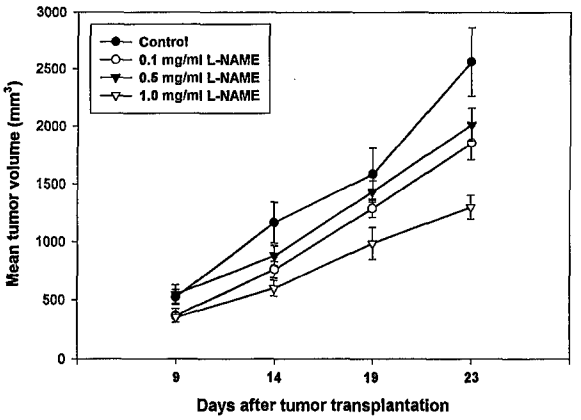

Figure 5. Effects of chronic L-NAME therapy given as two 4 day cycles (days 9–13 and days 19–23) at various concentrations of the drug in the drinking water, on the growth rate of primary tumors following a subcutaneous transplantation of 2.5×10^5 C3L5 tumor cells. Animals drank 3–4 ml water/day (n = 10–20). The data represent means ± SE. There was a dose-dependent decline in tumor growth which was significant at the highest dose (1 mg/ml) throughout the experimental period. *(Adapted with kind permission from Orucevic and Lala, Brit J Cancer 73: 189–196, 1996).*

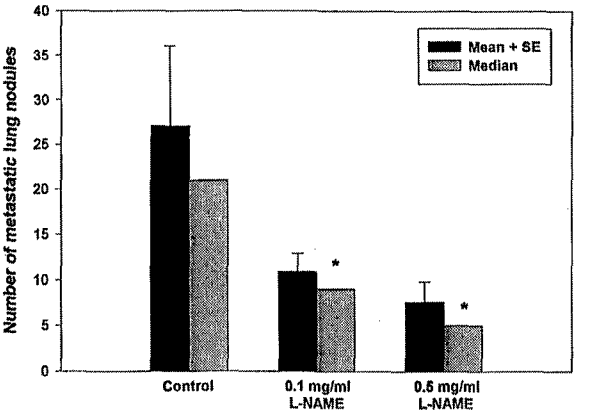

Figure 6. Effects of chronic L-NAME therapy (same dose and schedule as in Figure 5) on the number of spontaneous lung metastatic nodules of C3L5 tumor scored on day 23. There was a significant dose dependent reduction in lung metastasis. *(Adapted with kind permission from Orucevic and Lala, Brit J Cancer 73: 189–196, 1996).*

rine fibroblasts [52]. This phenotype can be detrimental to tumor cell survival. For example, engineered iNOS overexpression in a murine melanoma line abrogated tumorigenic and metastatic ability of these cells because of rapid apoptosis; these cells failed to survive even *in vitro* in the absence of NO-blocking agents [41]. However, tumor cells can vary widely in their susceptibility to NO-mediated apoptosis, from being highly susceptible to totally resistant. Reasons for such variation have not been fully explored. A variation in the genetic makeup can be suggested as one of the reasons. For example, susceptibility is provided by the expression of wild-type p53, and cellular ability to upregulate functional p53 in response to NO, whereas loss or mutation of p53 makes the cells resistant to NO-mediated apoptosis [49, 53, 54]. Furthermore, an overexpression of Bcl2 can protect tumor cells from NO-mediated apoptosis [55, 56].

VI. NO and tumor cell invasiveness: role of tumor-derived NO in the invasiveness of C3L5 cells

This was tested in an *in vitro* matrigel invasion assay [57], in which the invasive ability of tumor cells was determined by measuring the proportion of tumor cells transgressing a matrigel barrier during a 24–72 hour period, after blocking endogenous NO production or inducing additional NO production. Specifically, the effects of adding NOS inhibitors (NMMA, L-NAME in various concentrations) with or without excess L-arginine (which competes with the NOS inhibitors, abolishing their effects), or adding iNOS inducers (LPS in combination with IFN-γ) with or without NOS inhibitors were examined. The results can be summarized as follows. (i) Presence of NMMA or L-NAME reduced the invasion index; a parallel reduction in the level of NO production by the tumor cells measured by the $NO_2^- + NO_3^-$ levels in the medium was observed. (ii) Inclusion of excess L-arginine with NOS inhibitors abrogated the anti-invasive effects of the NOS inhibitors, attesting to the functional NOS specificity of the inhibitors. (iii) Inclusion of LPS and IFN-γ led to a major stimulation of NO production and a concomittant stimulaton of invasiveness. (iv) Inclusion of NOS inhibitors along with LPS and IFN-γ caused only a partial reduction in invasiveness, and LPS and IFN-γ-induced NO production. These findings clearly demonstrated that constitutive NO production by C3L5 tumor cells upregulated their

invasive ability. The invasion-stimulating effects of LPS and IFN-γ could be partially explained by a stimulation of NO production.

What are the mechanisms underlying the invasion-stimulating effects of NO in this tumor model? Earlier studies have shown that NO promotes degradation of articular cartilages by activating matrix metalloproteases (MMP's) in chondrocytes from numerous species [58, 59]. We hypothesized that NO leads to an alteration in the balance between the synthesis of MMP's and the synthesis of their natural inhibitors, i.e., tissue inhibitors of metalloproteases (TIMP's) in tumor cells. To test this hypothesis, the levels of MMP and TIMP mRNA expression were measured in these cells under different experimental conditions. C3L5 cells expressed the 72 kDa type IV collagenase (gelatinase A) and not the 92 kDa species (gelatinase B). They also expressed TIMP-1, TIMP-2 and TIMP-3. Phosphoimage analysis of Northern blots relative to the 18S RNA (loading controls) provided a measure of the mRNA expression under the various experimental conditions. The results are presented in Table 1.

Data presented in Table 1 show that (i) C3L5 cells expressed eNOS but not iNOS mRNA under native conditions, however, iNOS expression was induced in the presence of LPS and IFN-γ. This induction was upregulated by treatment of cells with NMMA, explained by a reduction of the NO-mediated negative feedback on the iNOS gene expression. (ii) NMMA treatment did not affect MMP-2 expression, but upregulated the expression of TIMP-2, and to a minor extent TIMP-3, indicating that the invasion-stimulating effects of endogenous NO are, at least in part, mediated by a downregulation of TIMP-2, and possibly TIMP-3. (iii) LPS and IFN-γ treatment upregulated MMP-2 and down-regulated TIMP-3, explaining the invasion-stimulating effects. Addition of NMMA to LPS and IFN-γ restrained the MMP-2 expression to normal level and only partially restored TIMP-3 expression, thus explaining the incomplete abrogation of LPS and IFN-γ stimulation of invasiveness with NOS inhibitors. These results indicated that LPS and IFN-γ-mediated stimulation of invasiveness is only partially explained by increased NO production, and that NO at higher levels can upregulate MMP-2 and downregulate TIMP-3. Indeed, we have observed that exposure of C3L5 cells to S-Nitroso-N-Acetyl-D, L-penicillamine (SNAP) (an NO donor) downregulates TIMP-3 mRNA (data not shown). Assuming that gene expression was directly related to the secretion of protein products, these results suggest that NO-mediated stimulation of invasiveness is due to an alteration in the balance between productions of the MMP's and TIMP's. Furthermore, NO has been shown to upregulate urokinase type plasminogen activator (uPA) in endothelial cells of post capillary venules during the process of NO-mediated stimulaton of angiogenesis [60]. Since uPA converts plasminogen to plasmin, which can activate numerous MMP's, this may represent another pathway of NO-mediated stimulation of matrix degradation.

Further studies of the relationship of NO to tumor cell invasiveness are needed using different tumor models. In K1735 murine melanoma cells, engineered overexpression of iNOS, leading to de-

Table 1. Image analysis* of Northern blots of mRNA expression in C3L5 cells

Transcript	Control	IFN-γ and LPS	IFN-γ and LPS + NMMA	NMMA
eNOS (4.5 kb)	1.0	1.0	1.0	1.0
iNOS (4.8 kb)	–	+	++	–
MMP-9 (2.5 kb)	–	–	–	–
MMP-2 (3.1 kb)	1.0	1.7	0.9	1.0
TIMP-1 (3.1 kb)	1.0	0.9	1.0	1.0
TIMP-2 (3.5 kb & 1.0 kb)	1.0	0.8	0.6	1.3
TIMP-3	1.0	0.3	0.6	1.2

* Standardized with 18S RNA used as a loading control. A positive expression in control cells is normalized to 1.0.
IFN-γ = 500 u/ml; LPS = 10 μg/ml; NMMA = 1 mM.

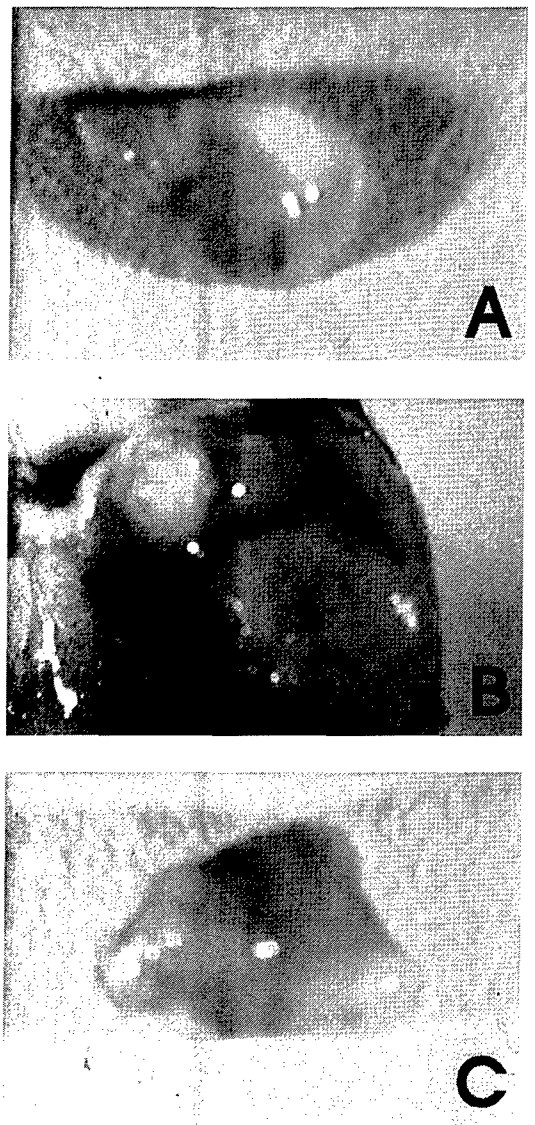

Figure 7. Gross morphology of matrigel implants in (A) matrigel alone implanted in D-NAME treated mice; (B) matrigel inclusive of tumor cells in D-NAME treated mice, and (C) matrigel inclusive of tumor cells in L-NAME treated mice. Note that implant A is mostly avascular, and implant B has grown in size and highly vascular, whereas implant C is smaller in size and less vascular in comparison with B.

creased tumor cell survival and tumorigenicity [41], has been reported to be associated with a down regulation of MMP-2 [61] owing to a downregulaton of MMP-2 promoter activity. It is thus possible that very high levels of endogenous NO may compromise invasive function of certain tumor cells, which are susceptible to NO-mediated cytotoxicity.

VII. NO and angiogenesis

VII. A. Roles of NO in angiogenesis under physiological conditions and during wound healing

Regulatory roles of NO in angiogenesis remain somewhat controversial. Pipili-Synetos *et al.* [62] suggested that NO is an endogenous inhibitor of angiogenesis. Using an angiogenesis assay which scores the number of blood vessels in a defined area of chick chorio-allantoic membrane (CAM assay), as well as tube formation by endothelial cells on matrigel, they found that Na-nitroprusside (an NO-donor) reduced and NOS inhibitors promoted basal angiogenesis. In contrast, Konturek *et al.* [63] found that inhibition of NO synthesis delayed healing of chronic gastric ulcers induced by acetic acid, by reducing local blood flow and angiogenesis at the periphery of the ulcers. Similarly, using both an *in vivo* angiogenesis assay with rabbit cornea and an *in vitro* assay which measures the growth and migration of capillary endothelial cells, Ziche *et al.* [64] showed that vasoactive substances such as substance P, or prostaglandin E $(PGE)_1$ stimulated angiogenesis in an NO-dependent manner, since it was blocked with NOS inhibitors NMMA, N^G-nitro-L-arginine (L-NNA) and L-NAME. NO donors such as Na-nitroprusside and glycerol trinitrate also stimulated endothelial cell migration. These authors have reported that one of the final pathways of NO-mediated angiogenesis is by upregulation of basic fibroblast derived growth factor (bFGF) in post capillary venule endothelial cells [60]. Angiogenic activity of human monocytes [65] and mitogenic activity of VEGF on coronary venular endothelium [66] have also been shown to be NO dependent. Indeed, NO was shown to be a downstream mediator of VEGF but not bFGF-induced angiogenesis [87].

It is possible that the conflicting data cited above on the role of NO in angiogenesis is due to the differences in levels of NO and NO scavengers in the microenvironment. Very high levels of NO may be

cytostatic or apoptosis-inducing for endothelial cells, whereas low to moderate levels of NO may promote endothelial cell migration, invasiveness and differentiation, either directly or by induction of angiogenic factors such as bFGF. A continuous remodelling of blood vessels, requiring highly localized endothelial cell death may be facilitated by high local endogenous NO levels (hot spots) in an embryonic tissue such as the CAM, which was utilized in the angiogenesis assay by Pipili-Syntos *et al.* [62]. This may account for the anti-angiogenic role of NO observed using this model, as opposed to the angiogenic role of NO in other models in which the basal level of NO may be low or negligible.

VII. B. Role of NO in tumor angiogenesis

Buttery *et al.* [34] suggested that iNOS expression by endothelial cells of the neovasculature of many experimental tumors promoted angiogenesis as well as blood flow in the vasculature, and thus sustained tumor growth. This suggestion has been reinforced by other investigators [67], and validated by Jenkins *et al.* [39] showing that the increased *in vivo* growth resulting from iNOS transfection of a human colon cancer cell line was associated with increased vascularity of the transplants in nude mice.

Table 2. NO and tumor biology: variables

Source of NO in the tumor:
Tumor cells (eNOS, nNOS, iNOS)
Tumor endothelium (eNOS, iNOS)
Tumor stroma, macrophages (iNOS)
Level of NO production by the tumor:
eNOS, nNOS: low to moderate
iNOS: moderate to high (high in an inductive environment)
Role of NO in tumor biology:
Genetic makeup of the tumor, for example:
wt $p53^{+}$: cytostasis, apoptosis (especially with high NO levels)
p53 null, mutant, inactive: resists NO mediated injury, and uses NO for tumor progression
Levels of NO:
High: cytostasis, apoptosis (in a susceptible genetic make up)
Low to moderate: promotion of tumor progression by increased angiogenesis, tumor microcirculation and tumor cell invasiveness

The latter findings have since been confirmed with other tumor cell lines transfected with iNOS, when the tumor cell lines had lost or mutated p53 [49].

Angiogenic role of NO in the C3L5 mammary tumor model (Jadeski, Hum, Orucevic and Lala, unpublished)

We gathered two types of evidence for the angiogenic role of NO in the C3L5 tumor model. (i) Tumors of the same age in mice subjected to NMMA therapy were compared with those in animals treated with vehicle alone for the incidence of blood vessels per unit area of the section of tumor tissue (Orucevic and Lala, unpublished). Tumors in NMMA-treated mice exhibited a significant reduction in the incidence of blood vessels. (ii) We designed a tumor angiogenesis assay by adapting the protocol of Kibbey et al. [68]. Rehydrated matrigel, which is liquid at 4 °C and solidifies at body temperature, was implanted subcutaneously in mice. The matrigel pellet stimulates the ingrowth of new blood vessels from the periphery of the implant, possibly because of the presence of angiogenic factors in conventional matrigel and by Kibbey *et al.* [68]. In our experiments, we replaced the conventional matrigel with growth factor-reduced matrigel which, on its own, stimulated little or no angiogenesis (Figure 7A) in C3H/HeJ female mice. However, inclusion of an appropriate number of C3L5 tumor cells in the matrigel was highly angiogenic. When animals were placed on chronic subcutaneous L-NAME therapy via osmotic minipumps shortly following the implantation, both the vascularity and the size of the tumor cell-inclusive implants declined (gross appearance shown in Figure 7C) as compared to the implants in control mice receiving vehicle alone or D-NAME therapy (Figure 7B). None of the therapies had any influence on the vascularity of the tumor cell-exclusive implants. These preliminary data suggest that an inhibition of NO production led to a reduced angiogenic ability of the tumor cells in the implants. Whether the decline in the growth of tumor cells was secondary to the angioreductive effects alone remains undetermined.

VIII. NO and host immune responses

A number of reports suggest opposing roles of NO in tumor immunity. Activated murine macrophages synthesize NO [69], which may partly mediate their cytotoxic activity against tumor cells [17, 50, 59], bacteria [16] and parasites [71]. Mills *et al.* [72] reported that ascites tumor growth in the mouse peritoneal cavity was associated with a reduced NO production by intratumor macrophages. Similarly, it has been reported that *in vitro* tumoricidal function of activated natural killer (NK) cells depends partly on their NO synthesizing ability [73–75]. In contrast, NO overproduction by rodent macrophages has been shown to suppress proliferation of T lymphocytes in response to antigens or mitogens [76, 77], and thus may hinder anti-tumor immune responses of T cells. Indeed, excessive NO production has been implicated in tumor-induced immunosuppression in rats [78]. We tested whether a potentiation of interleukin-2 (IL-2)-induced regression of C3L5 mammary tumors [37] resulting from L-NAME therapy can be explained, at least in part, by a potentiation of lymphokine activated killer (LAK) cell activation. We found that L-NAME treatment *in vivo* as well as *in vitro* markedly stimulated IL-2-induced generation of anti-tumor cytotoxicity of splenocytes in healthy as well as mammary adenocarcinoma-bearing mice; there was a parallel drop in IL-2-induced NO production *in vivo* and *in vitro* [79]. These results revealed that the IL-2-induced increase in NO production had a compromising effect on optimal LAK cell activation, which can be overcome by NO inhibition. In our hands, NO inhibitors added during the cytotoxicity assays had no detrimental effect on LAK cell mediated anti-tumor cytotoxicity. In summary, NO appears to be an important bioactive component of the cytotoxic pathways of anti-tumor effector cells, in particular macrophages. However, sustained NO release in the immune microenvironment is also detrimental to effector cell activation pathways, and thus suppress their anti-tumor function.

IX. Conclusions and suggestions

The above evidence suggest that NO may play opposing roles in tumor growth and metastasis. The precise role of NO produced by tumor cells or host cells in the tumor microenvironment may depend on two variables: (a) the level of NO production and (b) the genetic makeup of the tumor cells. Very high levels of NO can be detrimental to the survival of certain tumor cells as well as host cells because of NO-mediated cellular injury, cytostasis and apoptosis. Since high NO-producing tumor cell clones would likely delete themselves *in vivo*, it is reasonable to postulate that in most well established spontaneous tumors exhibiting a clonal heterogeneity, those producing low to moderate levels of NO or those capable of resisting NO-mediated cytostasis and apoptosis will survive and propagate. For these cells, tumor or host-derived NO may have a facilitating role for tumor progression by virtue of NO-mediated stimulation of invasiveness, angiogenesis and microcirculation within the tumor. These events may assume greater significance within the hypoxic regions of a tumor. In a solid tumor, hypoxia is known to provide a stimulus for angiogenesis by various pathways [80]. One of the mechanisms is the induction of vascular endothelial growth factor (VEGF) which has a hypoxia-responsive element in the promoter region of the gene. Hypoxia can also induce iNOS in a similar manner by activating the iNOS promoter via a hypoxia-responsive element [81]. The hypoxia-induced NO production may provide additional angiogenic and invasion-stimulating signals within a solid tumor.

The genetic makeup of tumor cells that may dictate susceptibility of resistance to NO-mediated injury remains to be completely investigated. One of the possible genetic determinants is the functional status of the p53 tumor suppressor gene. It has been shown that iNOS-transfected tumor cells expressing wild type functional p53 are vulnerable to NO-mediated cytostasis, because of an accumulation of p53 protein induced by endogenous NO [48, 49]. However, p53 accumulation eventually leads to a transcriptional transrepression of iNOS and thus inproves cellular survival [48]. Tumor cells in which p53 gene is deleted or mutated (causing a loss of

normal p53 function), on the other hand, can withstand NO-mediated cytostasis or apoptosis [49, 53, 54]. In addition, in the presence of endogenous NOS, p53-deficient or mutant tumor cells exhibit faster tumor growth and vascularity, when transplanted *in vivo* [49]. These observations led to the hypothesis that interaction of NO with p53 provides one mechanism of clonal selection of p53 mutant or p53 null cells which can utilize NO to their advantage for tumor progression *in vivo* [49, 82]. Since p53 mutation occurs in nearly half of all human cancers [83], this hypothesis predicts that NO would facilitate progression in a large proportion of human cancers. It remains to be seen whether a p53 dependent role is universal to all tumors and whether other tumor suppressor genes, e.g., Rb may interact with NO in a similar manner. The picture can be complicated further by other (nongenetic) mechanisms of protection from NO-mediated injury. For example, certain cells show acquired resistance to NO-mediated injury after prior exposure to NO [84, 85]. Nevertheless, availability of reliable genetic markers which can predict the specific role of NO in tumor biology will be highly valuable in determining the applicability of NOS inhibitors in treating specific tumors.

Table 2 presents a schema of the variables that may dictate the role of NO in tumor biology.

X. Key unanswered questions

1. Can the opposing roles of NO (on tumor progression vs. tumor regression) be explained by (a) the level of NO production, eg. high vs. moderate to low, (b) the ability of the tumor cell type to resist NO-mediated injury, or (c) both? Further experimentation using different tumor systems is needed to answer these questions. In particular, genetic determinants which may allow tumor cells to resist NO-mediated injury and exploit NO to their advantage deserve full exploration.
2. How universal is the phenomenon of NO-mediated promotion of tumor angiogenesis? This area needs further investigation using multiple tumor models with different NO producing abilities *in vivo*.
3. What are the effects of disrupting or down-regulating the eNOS gene in the high eNOS-expressing and highly metastatic C3L5 mammary tumor cells on their invasive, angiogenic and metastatic abilities? Conversely, what are the results of up-regulating eNOS in the low eNOS expressing, poorly metastatic C10 mammary tumor cells?

Acknowledgements

Studies from our laboratory reported in this article were supported by The National Cancer Institute of Canada with funds derived from The Canadian Cancer Society and the Department of the United States Army grant DAMD 17-96-6096 to PKL, and a post-doctoral fellowship from the Medical Research Council of Canada to AO.

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Cancer and Metastasis Reviews **17**: 127–142, 1998.

Role of nitric oxide in IL-2 therapy-induced capillary leak syndrome

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Key words: interleukin-2, capillary leak syndrome, nitric oxide, nitric oxide synthase inhibitors, NMMA, L-NAME, murine mammary adenocarcinoma

Abstract

Nitric oxide (NO) is a potent short-lived and short range bioactive molecule, which plays a key role in physiological and pathological processes including inflammation and cancer. Detrimental effects of excessive NO production during septic shock have been well recognized. We tested the hypothesis that 'capillary leak syndrome' following systemic interleukin-2 (IL-2) therapy resulted from a cascade of events leading to the induction of NO which, directly or indirectly, injured capillaries and caused fluid leakage. Our results provided the first direct evidence that the induction of active NO synthase (NOS) leading to the overproduction of NO is instrumental in IL-2-induced capillary leakage in mice and that successful blocking of this overproduction with chronic oral administration of NOS inhibitors can mitigate this leakage without interfering with the beneficial antitumor effects of IL-2 therapy. NO blocking agents can, in fact, improve IL-2-induced antitumor effector cell activation, as well as tumor regression. In our studies, NO blocking agents alone reduced the growth and metastasis of a murine mammary carcinoma, at least in part, by mitigating the invasion and angiogenesis-stimulating role of tumor-derived NO. Thus, NOS inhibitors may be useful in treating certain tumors and serve as valuable adjuncts to systemic IL-2 based immunotherapy of cancer and infectious diseases.

Introduction

During the last decade, use of systemic interleukin-2 (IL-2) became a major focus of interest in cancer immunotherapy because of IL-2 dependence of all anti-tumor effector cells i.e. T cells [1], natural killer (NK) cells [2] and macrophages [3, 4]. The success of high dose IL-2 therapy in metastatic murine cancers [5] soon led to human trials with IL-2 alone or in combination with *ex vivo* generated lymphokine-activated killer (LAK) cells [6–10], or in combination with chronic indomethacin therapy [11, 12] resulting in modest and variable success in renal cell carcinomas and melanomas.

Wide spread clinical use of systemic IL-2 based therapy, has been limited by a major side effect known as 'capillary leak syndrome'. It is characterized by retention of extravascular fluid, severe hypotension, and multiple organ system dysfunction [13, 14], often requiring cessation of IL-2 therapy. This syndrome has been documented in numerous species: humans [13, 15, 16], mice [17, 18], sheep [19–21] and rats [22].

Reported pathophysiological mechanisms underlying this syndrome include damage of endothelial cells by LAK cells [23, 24] or NK cells responding to IL-2 [25] or certain IL-2 induced cytokines e.g. interferon (IFN) γ [26] and tumor necrosis factor (TNF) α [27]. Injury to endothelial cells mediated by these cytokines has been recently linked with nitric oxide (NO) production [28, 29], because it was prevented with dexamethasone and inhibitors of NO synthesis. Severe hypotension observed during IL-2 therapy has also been recently attribut-

ed to NO production [30, 31]. NO is synthesized by many mammalian cells from the amino acid L-arginine, with the help of a family of enzymes called NO synthases (NOS) [32, 33]. It is a short lived biological mediator of many physiological functions. However, sustained overproduction of NO resulting from the induction of the inducible isoform of NOS (iNOS) may have pathological consequences including capillary damage because of cytotoxic action on endothelial cells [28, 29]. Vasodilation [34] and systemic hypotension due to NO production can indirectly cause pulmonary hypertension, and the increased pulmonary capillary pressure [13] can lead to fluid leakage in the lungs. Thus, NO may have a major role in the pathogenesis of IL-2 induced capillary leakage. In this paper, we shall briefly review the IL-2 based cancer therapies and possible pathways of IL-2 therapy induced capillary leakage. Since IL-2 therapy induces production of LAK cells [16, 35], IFNγ [36], TNFα [15, 37], and NO [30, 31], we shall discuss the independent as well as interdependent roles of these multiple factors. We shall show that NO overproduction occurs at the later part of a cascade responsible for this syndrome and that appropriate administration of NOS inhibitors can not only overcome the syndrome but also improve antitumor effects of IL-2 therapy.

Systemic IL-2 in tumor immunotherapy

A. Biology of IL-2: Tumor therapy with IL-2 as a single agent

T cell growth factor (later named as IL-2) was initially identified in the supernatant of phytohemagglutinin-stimulated normal human lymphocytes that supported the growth of T cells in culture of normal human bone marrow [38]. IL-2 has since been characterized as a 133 amino acid polypeptide of 15,500 daltons [39], in humans it is encoded by a single gene [40] on chromosome 4. Recombinant IL-2 has been obtained by inserting the IL-2 gene from cultured leukemic cells [41] or from normal peripheral blood lymphocytes [42] in *Escherichia coli*. This form of recombinant IL-2, although non-glycosylated, has biological activity *in vitro* and *in vivo* identical to that of native IL-2.

The structure of the IL-2 receptor consists of 3 peptide chains (α, β and γ); the genes encoding these chains have been cloned and characterized [43, 44]. The α chain alone provides a receptor of low affinity. Intermediate and high-affinity receptors are produced by β/γ heterodimer and α/β/γ heterotrimer, respectively, in which the β chain is critical for signal transduction [45]. IL-2 receptor expression has been variably found on the surface of T cells, NK cells [2, 46], macrophages [3, 4, 47], oligodendroglial cells [48], epidermal Langerhans cells [49], B cells [50], and certain tumor cells lines derived from melanomas and squamous cell carcinomas of the head and neck [51].

The tumoricidal potential of all immune effector cells including T cells [1], NK cells [2, 52, 53], and macrophages [3, 4] can be stimulated with IL-2. Lymphocytes cultured in the presence of high dose IL-2 lead to the activation of NK cells and T cells, providing a heterogenous population of cytotoxic cells with a broad spectrum of antitumor cytotoxicity, known as LAK cells which are capable of killing syngeneic as well as allogenic tumor cells [54]. This knowledge provided the impetus for systemic IL-2 therapy of cancer.

Rosenberg *et al.* [5] were the first to show that systemic administration of IL-2 resulted in regression of pulmonary metastasis in mice by activation of LAK cells *in vivo*. These findings led to the application of IL-2 therapy in human cancers, revealing that highest tumor regression occurred in melanomas and renal cell carcinomas [16, 55, 56].

B. Systemic IL-2 in combination with LAK cells in tumor therapy

Rosenberg's group observed that a combination of systemic IL-2 therapy with infusion of LAK cells generated *in vitro* had significantly higher antitumor activity in mice than IL-2 therapy alone [57, 58]. Intravenous IL-2 therapy in combination with LAK cells was then applied to treat human patients with solid tumors. Autologous lymphocytes were obtained from cancer patients by repeated leukapheresis, cultured in the presence of IL-2 to generate LAK cells, and reinfused into the patients together with IL-2 [16]. This treatment resulted in the regression of tumors in some patients for whom no

other effective therapy was available [35, 59]. However, it was soon apparent that the therapeutic benefit derived from this combination therapy was not greater than that from IL-2 therapy alone [7].

C. IL-2 in combination with tumor infiltrating lymphocytes in tumor therapy

Lymphocyte-trafficking studies with radiolabeled LAK cells generated from blood or splenic lymphocytes showed that LAK cells did not localize at tumor metastatic sites but were trapped in the lungs and later in the liver. However, lymphocytes retrieved from the tumor and expanded with IL-2 showed some selectivity for migration to the tumor metastatic site after infusion *in vivo* [60]. These 'tumor infiltrating lymphocytes' (TIL) were expanded *in vitro* [61] for adoptive transfer. When TILs were infused along with IL-2 in patients with melanoma or renal cell carcinoma, responses were higher relative to IL-2 treatment alone or IL-2 combined with LAK cells [61–63].

D. IL-2 in combination with chronic indomethacin therapy in tumor treatment

Lala *et al.* [64] observed that natural killer cells were progressively inactivated in the tumor-bearing host with increasing tumor burden. This inactivation was caused by a high level of prostaglandin E_2 (PGE_2) produced by host macrophages [65] as well as certain tumor cells [66]. PGE_2 has been shown to suppress lymphocyte proliferation [67] and activation of all antitumor killer cell lineages [4]. These effects, at least in part, explained the promotion of metastatic ability of tumors by PGE_2 [68]. The PGE_2-mediated inactivation of effector cells was attributed to inhibition of IL-2 production [69] and a down regulation of IL-2 receptors on the surface of all killer cell lineages [70].

Based on these findings, Lala's group started an immunotherapy protocol combining systemic IL-2 with chronic oral administration of indomethacin [71], a drug that inhibits prostaglandin production [67]. Chronic indomethacin therapy had antitumor and antimetastatic effects [72, 73], and substantially restored natural killer cell function [73] in murine tumor models. However, this therapy alone was unable to eradicate advanced metastases [71], possibly because of inadequate IL-2 production *in vivo*. Chronic indomethacin therapy (given in the drinking water) when combined with systemic injections of IL-2 resulted in permanent regression of B16F10 melanoma metastases in the lungs of a large proportion of animals [74]. Reactivation of AGM-1 + and Thy-1 +/– killer lymphocytes *in situ* accounted for the therapeutic benefit, since depletion of these cells *in vivo* abrogated the therapeutic effects. Similar eradication of metastases was also achieved in C3-L5 mammary adenocarcinomas [75] and human melanomas grown in nude mice [76]. This combination therapy was then applied in a phase 2 human trial of advanced melanoma and renal cell carcinoma patients resulting in good objective responses [11, 12], comparable with the higher ranges in the success rates reported in the other IL-2 based therapy trials [77]. Interestingly, the toxicity was manageable in a general oncology ward without the need for vasopressor agents often used in other IL-2 trials [77], and some melanoma patients responded to indomethacin therapy alone [78].

Capillary leak syndrome due to systemic IL-2 therapy

Initially, it was believed that the efficacy of IL-2 in the therapy of cancer improved as a function of the IL-2 dose administered [56, 59]. Although true for animal models, this association was very weak in a controlled study in renal cell carcinoma patients, receiving high or low-dose of intravenous IL-2 [14], and undetectable in a study in renal cell carcinoma patients using indomethacin in combination with IL-2 [11, 12, 79]. However, dose-related toxicity was observed in most trials and still remains a major obstacle to systemic IL-2 based therapy. Capillary leak syndrome is the most serious side effect of moderate to high doses of IL-2 observed in many species [13, 15, 18, 20–22]. There is an increase in microvascular permeability causing marked accumulation of extravascular fluid in all organ systems and hypotension, often requiring treatment with intravenous fluids and vasopressor agents [81]. Retention of extravascular fluid results in rapid weight gain of up to 20%, manifested by peripheral edema, pleural effu-

sion and ascites [13, 14]. Occasionally, life threatening pulmonary edema, respiratory or cardiac failure, and neurological abnormalities resulting in coma (due to edema of the brain) may develop during IL-2 therapy, requiring cessation of the therapy [13, 14]. Interestingly, symptoms of capillary leakage begin to reverse within 24 h of cessation of IL-2 therapy and usually completely disappear within a few days [14]. Capillary leak syndrome has been observed with IL-2 therapy alone and IL-2 therapy in combination with LAK cells or TIL. A less severe form of the syndrome has also been noted with IL-2 therapy in combination with indomethacin therapy.

Several studies have combined IL-2 with other agents to ameliorate the capillary leakage. However, the added drugs also blocked or reduced the beneficial antitumor effects of IL-2. Corticosteroids [17], which suppress inflammatory responses and induction of NO [82], and asialo-GM-1 antibody, which depletes LAK cells [18], both fall in this category. Puri *et al.* [83] reported that IL-1α reduced IL-2-induced capillary leakage but did not improve animal survival. Welbourn *et al.* [84] reported that certain cyclopeptides (e.g. antamanide and phalloidin), reduced IL-2-induced edema in the rat, presumably by causing cytoskeletal changes in neutrophils with consequent suppression of endothelial injury by thromboxane B_2. Influence of these agents on the antitumor effect of IL-2 remains unknown. Further studies were therefore required to identify substances that can ameliorate capillary leakage without compromising the anti-tumor effects of IL-2.

Based on the observations that systemic IL-2 therapy in combination with chronic indomethacin therapy in advanced melanoma and renal cell carcinoma patients [11, 12] was associated with less severe IL-2 toxicity than reported in the case of other IL-2-trials, we tested in a mouse model whether PGE_2 played any role in the IL-2-induced capillary leakage [85, 86]. Our results revealed that addition of chronic indomethacin treatment markedly improved the antitumor effects of IL-2 therapy, but was unable to ameliorate the IL-2-therapy – induced capillary leakage.

A. Possible mechanisms of IL-2 induced capillary leak syndrome

At least one of two conditions must be satisfied to cause capillary leakage: capillary endothelium must be damaged or the capillary pressure increased. Five mechanisms have been proposed by which IL-2 therapy can induce capillary leakage.

(i). IL-2 induces LAK cells to adhere to and later damage endothelial cells.

(ii). IL-2 induces NK cells to adhere to and damage endothelial cells.

(iii). Endothelial cells are damaged by TNF-α produced by IL-2-activated leukocytes.

(iv). Changes in endothelial cell architecture are caused by IFNγ produced by IL-2-activated leukocytes.

(v). IL-2 directly or indirectly induces NO production which is toxic for endothelial cells. In addition, NO, because of its vasodilatory role, leads to systemic hypotension which indirectly causes pulmonary hypertension resulting in an increase in pulmonary capillary pressure and thus pulmonary edema.

A.1. LAK cells and capillary leak syndrome. LAK cells have been shown to adhere to endothelial cells and cause their lysis *in vitro* [23, 24, 87]. Kotasek *et al.* [23] proposed that the dense granules secreted by LAK cells, which contain serine esterase I (an enzyme with high proteolytic and cytolytic activity, caused breaches in the endothelial cell membranes. Observations on cultured endothelial cells led Savion *et al.* [88] to propose that LAK cells migrated through and ruptured endothelial cell tight junctions. Once they reached the basement membrane and the subendothelial matrix, LAK cells would degrade the matrix by producing matrix-degrading enzymes. These events, with or without endothelial cell lysis, would result in capillary leakage. The hypothesis of LAK cell mediated capillary injury is substantiated by the findings that LAK cell depletion *in vivo* by treatment with asialo-GM-1 antibody in mice ameliorated IL-2 therapy induced capillary leakage [18]. However, this treatment also abrogated antitumor effects of IL-2.

A.2. NK cells and capillary leak syndrome. Aronson

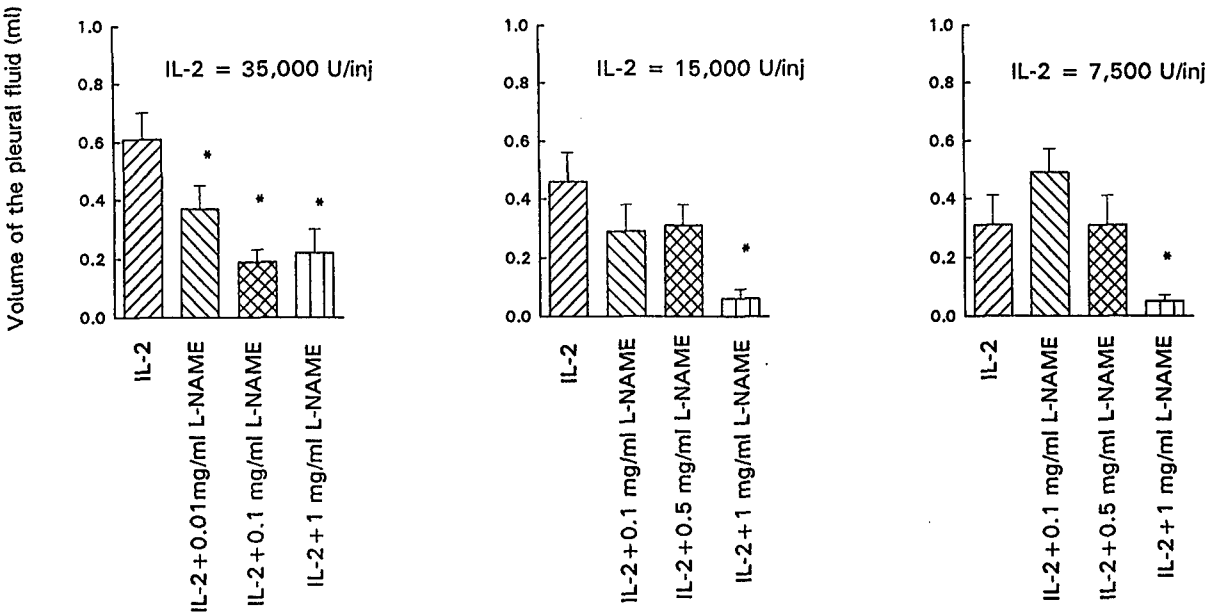


Figure 1. Pleural effusion after IL-2 and L-NAME therapy in healthy mice. Data represent mean ± SE (n = 5). * indicates significant difference from IL-2 treatment ($p < 0.05$).
L-NAME (0.01, 0.1 or 1 mg/ml of drinking water) significantly ($p < 0.05$) reduced IL-2 (35,000 U/inj i.p., every 8 h, 10 inj. total) induced pleural effusion. Significant reduction ($p < 0.05$) of pleural effusion induced by lower IL-2 dose (15,000 U/inj or 7,500 U/inj) was noticed only with high L-NAME dose (1 mg/ml of drinking water). Neither control (untreated) nor L-NAME alone treated mice showed any pleural effusion (data not shown). *Reproduced with kind permission from Orucevic and Lala, J Immunother., 18: 210–220 1996 Lipincott Raven Publishers.*

et al. [25] showed that IL-2 can induce NK cells to adhere to human endothelial cells in culture. These authors implied that vascular leakage induced by IL-2 resulted from NK cell mediated endothelial cell injury. However, there has been no direct evidence of NK cells causing endothelial cell damage *in vivo.*

A.3. TNFα and capillary leak syndrome. IL-2 therapy activates leukocytes (monocyte-macrophage in particular) to produce TNFα [15, 37]. Several authors have reported controversial findings about the ability of TNFα to damage endothelial cells. Collins *et al.* [89] reported that TNFα activated human endothelial cells to express class 1 HLA antigen, suggesting that TNFα made them prone to cytolytic T lymphocyte mediated injury. Kahaleh *et al.* [27] showed that TNFα inhibited endothelial cell growth in culture, and at high concentrations, induced endothelial cell lysis.

In 1990, Doukas and Pober [90] reported that TNFα led to endothelial cell 'activation', which was enhanced further by IFNγ. 'Activation' was indicated by appearance of new morphologic, antigenic and functional characteristics of endothelial cells. Increases in specific endothelial cell surface molecules like ELAM-1 (endothelial leukocyte adhesion molecule 1) or ICAM (intercellular cell adhesion molecule) were observed by these authors after stimulation by TNFα and IFNγ. IL-6 production in response to TNFα was observed by Leewenberg *et al.* [91]. Endothelial cell activation and increased adhesiveness for leukocytes were implied to play a role in increased capillary permeability.

In contrast, Mier *et al.* [92], reported that TNFα and IFNγ activated endothelial cells and increased the binding of CD16 + lymphocytes to endothelial cells in culture, but that the lymphocyte binding was not responsible for increased capillary permeability. In fact, these authors reported that TNFα and IFNγ protected endothelial cells from LAK cell-mediated injury.

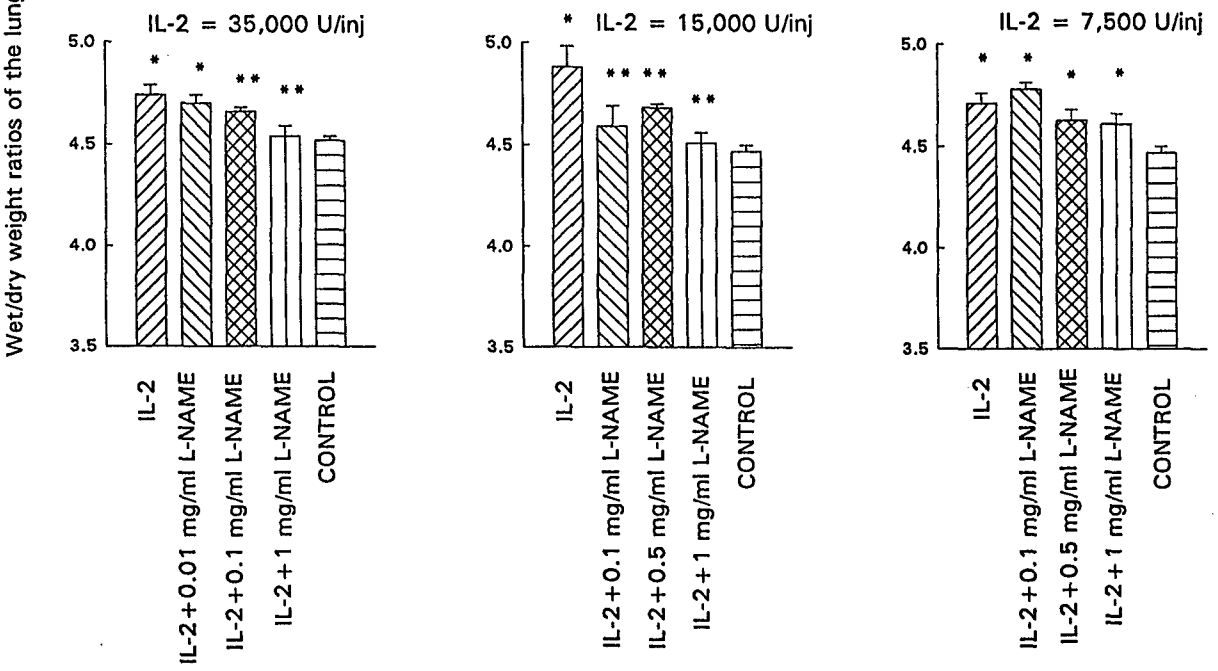


Figure 2. Water content of the lungs after IL-2 and L-NAME therapy in healthy mice. Data represent mean ± SE (n = 5). * indicates significant difference from control ($p < 0.05$). ** indicates significant difference from IL-2 treatment ($p < 0.05$). IL-2 (35,000 U/inj or 15,000 U/inj i.p., every 8 h, 10 inj total) induced pulmonary edema was significantly ($p < 0.05$) reduced with addition of L-NAME (0.1 mg/ml or more) in a dose dependent manner, being abolished at a dose of 1 mg/ml. Low dose of IL-2 (7,500 U/inj) also induced pulmonary edema, but addition of L-NAME did not have any significant effect. *Reproduced with kind permission from Orucevic and Lala J Immunother., 18: 210–220, 1996 Lupincott Raven Publishers.*

A.4. Changes of endothelial architecture induced by IFNγ. IFNγ appears in the blood of cancer patients within 6 hours after administration of IL-2 [15]. Cytotoxic activity of IFNγ is well known, but its possible role in IL-2 induced capillary leak syndrome remains obscure. Montesano *et al.* [26] showed that certain lymphokines could alter human endothelial cell architecture *in vitro*. IL-2 had no effect, and IFNγ had only a marginal effect. Combination of IL-1 and IFNγ completely changed the appearance of endothelial cells. They became elongated with many 'dendrite like' processes, and there were changes in cytoskeletal structure.

A causal relationship between changes in endothelial cell morphology induced by these lymphokines *in vitro* and in the capillary leak syndrome *in vivo* remains to be established. In fact, Puri *et al.* [83] reported that administration of recombinant IL-1 *in vivo* reduced IL-2 induced vascular leakage in the lungs of mice. These authors could not explain the failure of IL-1 to increase survival of mice treated with IL-2 or with IL-2 and IFNγ.

A.5. NO and capillary leak syndrome. Based on the findings that NO can be produced by activated macrophages after treatment with endotoxin, IFNγ or certain other cytokines [93, 94], Kilbourn and Belloni [95] investigated the effects of IFNγ, TNFα, IL-1, IL-2 and endotoxin on the production of NO by endothelial cells. They showed that culture of murine brain endothelial cells produced NO in response to various combinations of cytokines. They speculated that endothelium-derived NO played a role in the development of hypotension in patients treated with IL-2 or TNFα. In support of this hypothesis, Kilbourn *et al.* showed that therapy with N^G-Methyl-L-Arginine (NMMA, an inhibitor of NO synthesis) protected dogs against hypotension induced by TNFα and endotoxin [96, 97], as well as IL-2 [80].

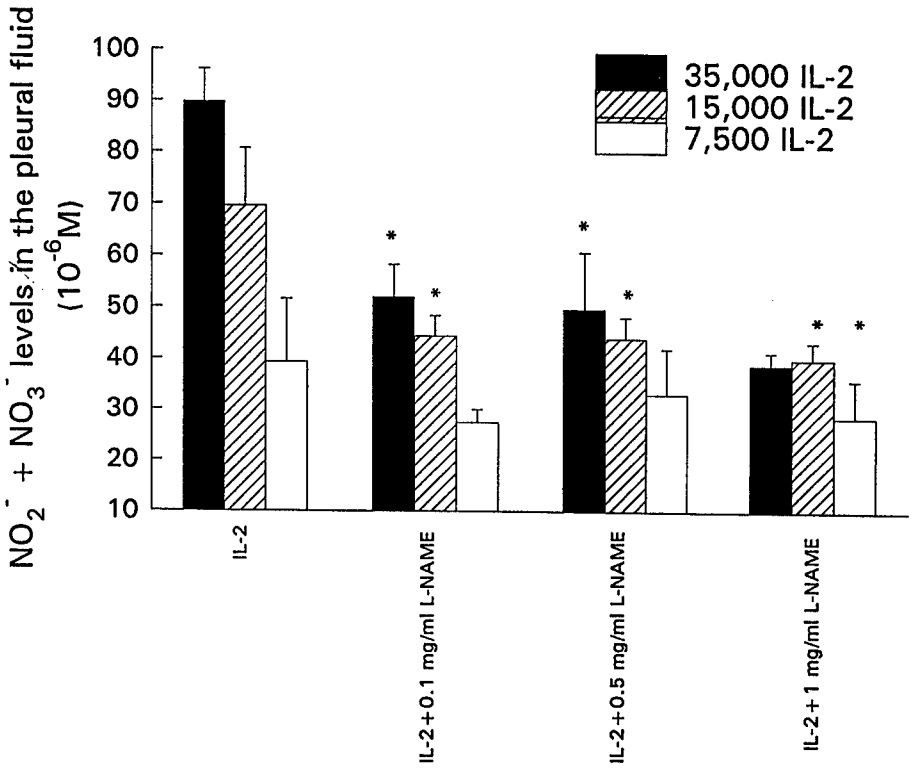
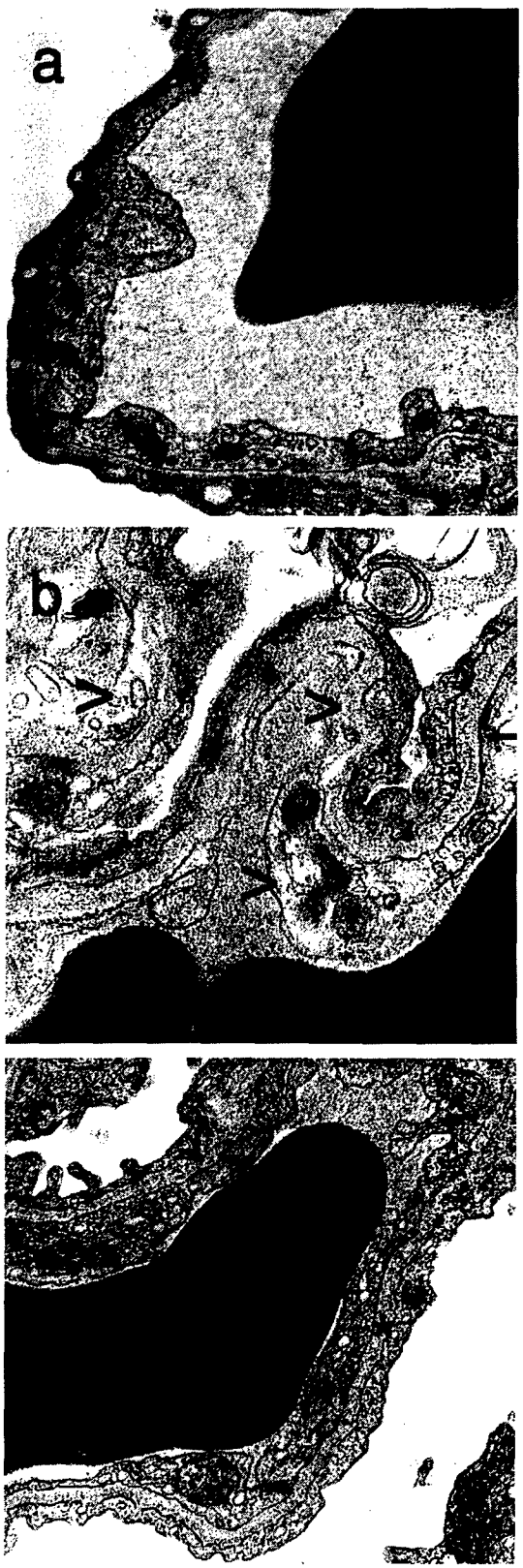


Figure 3. Nitrite + nitrate levels in the pleural effusion after IL-2 and L-NAME therapy in healthy mice ($10^6 \times M = \mu M$). Data represent mean ± SE (n = 3–5, each done in duplicate). * indicates significant difference from IL-2 treatment ($p < 0.05$). IL-2 (15,000 U/inj or 35,000 U/inj, i.p., every 8 h, 10 inj. total) induced dose dependent increases in nitrite + nitrate levels in the pleural effusion were significantly ($p < 0.05$) reduced with addition of L-NAME (0.1 mg/ml or more). L-NAME did not have any effects on the nitrite + nitrate levels induced by low IL-2 dose (7.500 U/inj). *Reproduced with kind permission from Orucevic and Lala J Immunother., 18: 210–220, 1996 Lipincott Raven Publishers.*

Increased levels of the final metabolites of NO (nitrates and nitrites) [82, 98] have been reported in human cancer patients receiving IL-2 therapy [30, 31, 99]. NO induction may be an indirect result of IL-2 therapy due to an induction of IFNγ and TNFα [15, 37]. Endothelial injury mediated by both of the cytokines has been linked with NO production [28, 29]. NO can contribute to capillary leakage by direct or indirect mechanisms. First, NO has been shown to mediate cytotoxicity in endothelial cells [28, 29] and thus cause a loss of integrity of the capillary lining. Second, high NO levels can indirectly enhance the capillary leakage in the lungs. It causes systemic hypotension [100] which in turn can indirectly cause pulmonary hypertension and thus increased pulmonary capillary pressure leading to further fluid leakage in the lungs.

Capillary leak syndrome results from numerous simultaneous or sequential events induced by IL-2 therapy: a hypothesis

In view of the literature reviewed earlier, it is reasonable to suggest that an increase in capillary permeability is caused by multiple factors initiated by high doses of IL-2. These factors operate by causing damage to capillary endothelial cells and/or by increasing capillary pressure. Capillary leak syndrome may result from numerous simultaneous or sequential events: 1) IL-2 induces LAK cell activation *in vivo* and promotes their adhesion to and subsequent cytotoxicity to endothelial cells; 2) IL-2 induces high levels of IFNγ which can change the cytoarchitecture of endothelial cells, making the endothelial lining more prone to leakage; 3) high levels of TNFα produced by IL-2-activated leuko-



cytes induces endothelial cell activation and adhesiveness for leukocytes and may play a role in increased capillary permeability; 4) NO induction at high levels remains at the end of the cascade of events induced by IL-2 therapy (e.g. production of TNFα and IFNγ) and plays a major role in capillary leakage both directly and indirectly, as discussed above.

Evidence for the central role of NO in the development of IL-2 therapy-induced capillary leakage in mice and its mitigation with NOS inhibitors

We conducted a series of studies to examine the role of NO in the pathogenesis of capillary leakage resulting from systemic IL-2 therapy in healthy and mammary adenocarcinoma-bearing C3H/HeJ mice. We measured IL-2 therapy-induced capillary leakage (pleural effusion, pulmonary edema and water retention in the spleen and the kidneys), NO production *in vivo* and the influence of treatment with NOS inhibitors (NMMA and N^G-Nitro L-Arginine methyl ester – L-NAME) on these parameters. Influence of these two inhibitors on IL-2 therapy-induced regression of the primary tumors and their lung metastases was also examined. In addition, the effects of these NOS inhibitors alone on

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Figure 4. Ultrastructure of the lungs of mice given IL-2 or IL-2 + L-NAME therapy. a = control b = IL-2: c = IL-2 + L-NAME; magnification × 20,000. Therapies were given in the following manner: IL-2 was given in a dose of 15.000 U/inj,i.p., every 8 h, 10 injections total; L-NAME was given in drinking water starting 1 d before IL-2 therapy.

Basement membrane is thick (→) and discontinuous in IL-2 treated mice. Endothelial as well as pneumocyte type I are severely damaged. There is also swelling of endothelial cells as well as pneumocyte type I. > indicates an area of blood-air barrier showing such damage. Addition of L-NAME therapy restored the ultrastructural integrity of alveoli and endothelium. Basement membrane is continuous and thin at the thin part of the capillary (*) in IL-2 + L-NAME treated animals. Endothelial cells, although in some cases remain swollen, are never detached from their basement membrane in these mice. *Reproduced with kind permission from Orucevic et al., Lab. Investigation, 76(1): 53–65, 1997, The United States and Canadian Academy of Pathology Inc.*

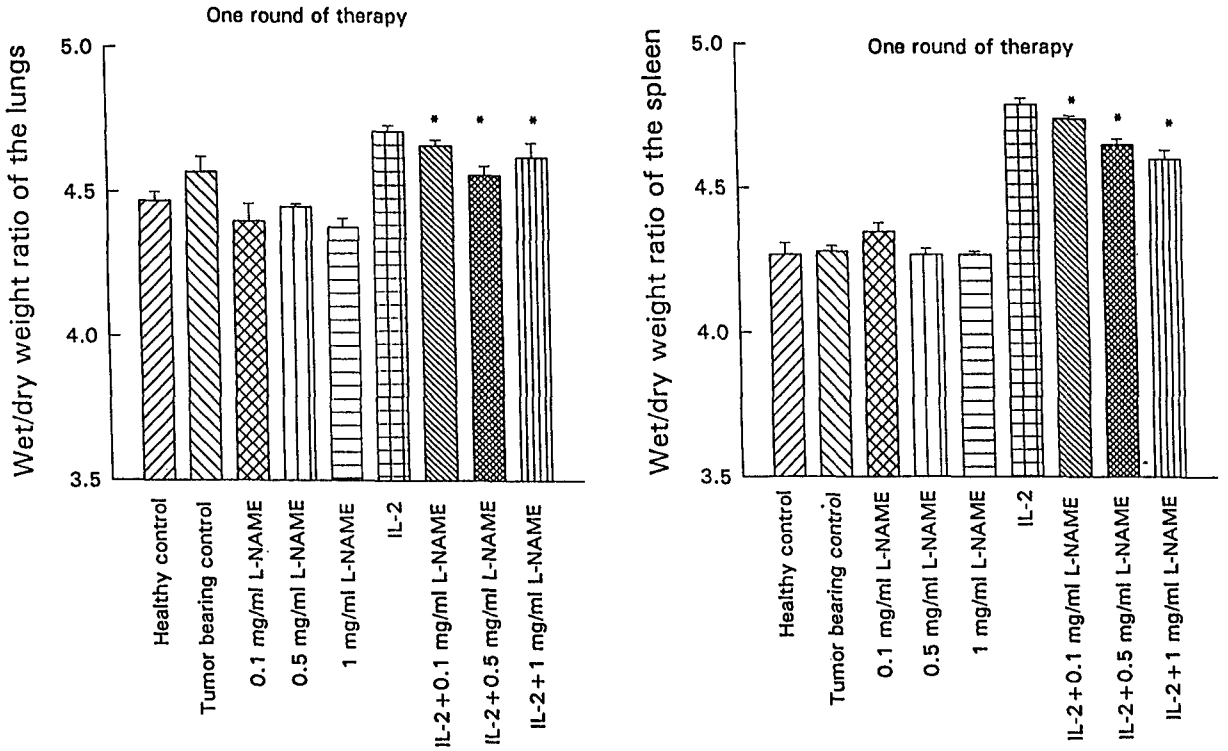

Figure 5. Water content of the lungs and spleen after IL-2 and L-NAME therapy in tumor-bearing mice. (left: lungs, right: spleen). Data represent mean ± SE (n = 10). Therapies were given in the following manner: IL-2 was given i.p. in a dose of 15,000 U/inj, every 8 h, 10 injections total, started 10 d after sc. inj of 250,000 C3–L5 mammary adenocarcinoma cells; L-NAME was given in drinking water starting on d 9 after tumor inoculation. * Addition of L-NAME significantly ($p < 0.05$) reduced IL-2 induced pulmonary edema after the first round of therapy.

* Addition of L-NAME significantly ($p < 0.001$) decreased IL-2 induced water retention in the spleen in a dose dependent manner after the first round of therapy. *Reproduced with kind permission from Orucevic and Lala, Br. J Cancer, 73: 189–196, 1996, Stockton Press, Hampshire, UK.*

mammary tumor growth and metastases were evaluated. Since L-NAME potentiated tumor-reductive effects of IL-2 therapy simultaneously with a reduction of IL-2-induced NO production *in vivo*, further experiments were designed to test whether L-NAME had a potentiating effect on IL-2 induced activation of antitumor effector cells *in vivo* and *in vitro*. This was tested by measuring antitumor cytotoxicity of splenocytes of healthy or tumor-bearing mice subjected to IL-2 ± L-NAME treatment *in vivo* and *in vitro*.

We initially tested whether treatment with NMMA can ameliorate IL-2 therapy-induced capillary leak syndrome in healthy or tumor-bearing mice without compromising the antitumor effects of IL-2 therapy [101]. We found that intraperitoneal IL-2 therapy caused substantial capillary leakage, both in healthy and tumor-bearing mice, as well as a substantial rise in NO production *in vivo* (measured in the serum and pleural effusion) in an IL-2 dose-dependent manner. Subcutaneously administered NMMA, when combined with IL-2 therapy, failed to ameliorate IL-2-induced capillary leakage in both groups of mice, and was also inadequate in significantly reducing IL-2 induced rise in NO production *in vivo*. It did not compromise anti-tumor effects of IL-2. In mammary adenocarcinoma bearing mice, subcutaneous NMMA therapy alone reduced tumor growth, spontaneous pulmonary metastasis and tumor-induced pulmonary edema.

This prompted us to test the effects of continuous oral administration of NMMA in healthy mice sub-

jected to IL-2 therapy. A substantial drop in NO production and capillary leakage was noted in these mice [101]. Since NO-blocking agents protected against IL-2-induced hypotension [80, 100], it was reasonable to expect that NMMA should also prevent IL-2 induced fluid leakage. It was evident that NMMA fulfilled this expectation only when given orally but not subcutaneously. This may be because the continuous oral administration of the drug was effective in blocking the rise in serum NO levels induced by IL-2 therapy, whereas the subcutaneous administration, in spite of repeated delivery, was inadequate in fully blocking NO production [101]. We suggested that the route of administration, as well as scheduling, were important determinants of therapeutic efficacy of NO inhibitors in the mitigation of IL-2 induced capillary leakage. This contention was supported by our findings that another NOS inhibitor, L-NAME also succeeded in mitigating IL-2 induced capillary leakage in healthy mice when given orally, but the benefits were only partial when given subcutaneously [102]. Similarly, attenuation of IL-2-associated capillary leakage was observed in another murine model by Samlowski *et al.* [103] when an NO inhibitor was given continuously in an osmotic minipump, whereas no effect on the IL-2-induced capillary leakage was noted by Leder *et al.* [104] when the NO inhibitor was given subcutaneously.

We did not test oral NMMA therapy in tumor-bearing mice, since L-NAME, another potent and less expensive NO inhibitor was soon available. Thus, we first tested whether L-NAME given chronically in the drinking water was effective in preventing capillary leakage induced by IL-2 therapy in healthy mice. We found that L-NAME was effective in preventing capillary leakage (pleural effusion – Figure 1, pulmonary edema – Figure 2 and water content of the spleen – not shown) induced by IL-2 therapy in healthy mice, and reduced IL-2-induced mortality when the IL-2 dose was not very high [102]. NO production appeared to be a strong determinant of the severity of this syndrome, because L-NAME treatment had a parallel effect in ameliorating the IL-2-induced capillary leakage and rise in NO production (Figure 3). A subsequent study [105] was designed in healthy C3H/HeJ mice

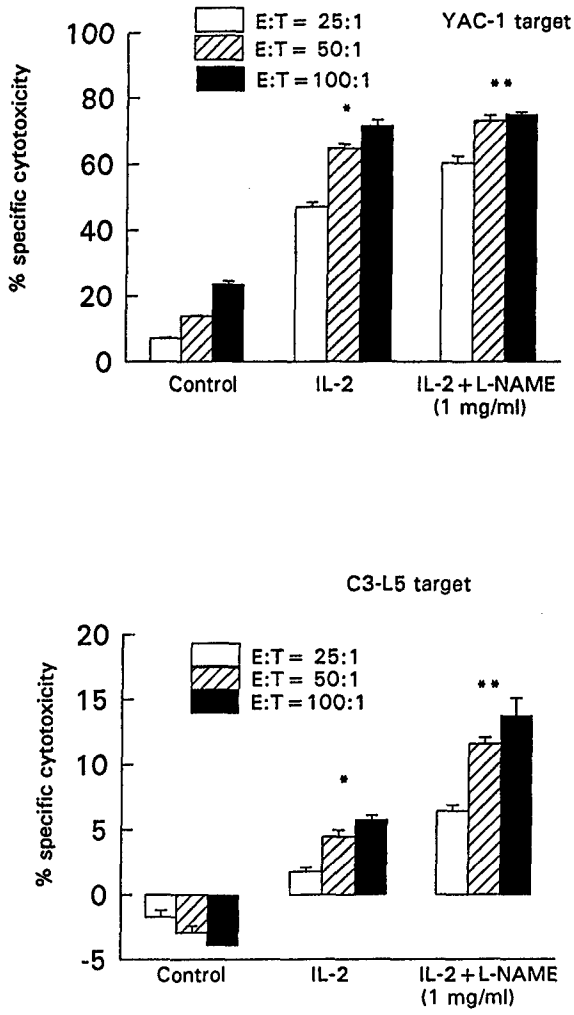

Figure 6. In vivo killer cell generation in healthy mice after IL-2 ± L-NAME therapy. Data represent mean ± SE (every effector: target ratio done in triplicate). * IL-2 therapy significantly ($p < 0.05$) improved splenocyte cytotoxicity (all three effector: target ratios combined) against NK sensitive-YAC-1 target and NK resistant – C3–L5 target. ** Addition of L-NAME therapy significantly ($p < 0.05$) enhanced IL-2 induced splenocyte cytotoxicity (all three effector: target ratios combined) against NK sensitive and NK resistant targets. *Reproduced with kind permission from Orucevic and Lala, Cellular Immunol., 169: 125–132, 1996, Academic Press, New York, USA.*

to (a) identify the tissue source of NOS activity and NOS protein induced by IL-2 therapy; (b) identify the histological nature of structural damage to the lungs during IL-2 therapy-induced pulmonary edema and (c) test whether the addition of L-NAME

therapy reduced the increase in NOS activity and IL-2-induced structural damage to the lungs. Morphological studies revealed that IL-2 therapy led to the induction of iNOS protein in numerous tissues, including the vascular endothelium, muscles of the anterior thoracic wall and splenic macrophages [105]. Biochemical studies revealed a positive association of high NOS activity in the lungs and the anterior thoracic wall with the presence of pulmonary edema, pleural effusion and structural damage to the lungs and its capillaries in IL-2 treated mice. Addition of L-NAME completely abolished the NOS activity, but not necessarily iNOS expression. It also reduced IL-2-induced pulmonary edema and pleural effusion, and significantly restored structural integrity of the lungs identified by light and electron microscopy (Figure 4) [105]. Thus, high tissue activity of IL-2-induced iNOS enzyme played a crucial role in the pathogenesis of IL-2-induced capillary leak syndrome.

Next, we tested whether L-NAME can prevent IL-2-induced capillary leakage in mammary adenocarcinoma bearing mice without compromising the therapeutic benefit of IL-2 [106]. In tumor-bearing mice, oral L-NAME therapy alone produced significant anti-tumor and anti-metastatic effects, similar to the effect noted earlier with NMMA therapy. L-NAME in combination with IL-2 therapy succeeded in ameliorating IL-2-induced as well as tumor-induced capillary leakage in tumor-bearing mice (Figure 5), and potentiated the tumor-reductive function of IL-2 [106]. Therefore, we tested whether a potentiation of IL-2-induced tumor regression by L-NAME therapy can be explained by a potentiation of LAK cell activation [107]. We found that L-NAME treatment *in vivo* as well as *in vitro* markedly stimulated IL-2-induced generation of antitumor cytotoxicity of splenocytes of healthy (Figure 6) as well as mammary adenocarcinoma-bearing mice (not shown), concomitant with a drop in IL-2-induced NO production *in vivo* and *in vitro*. These results revealed that the IL-2-induced increase in NO production had a compromising effect on optimal LAK cell activation, which can be overcome by NO inhibition with L-NAME therapy [107].

The above results provided the first direct evidence that NO is instrumental in IL-2-induced capillary leakage and that an NO blocking agent such as L-NAME can mitigate this leakage without interfering with the beneficial anti-tumor effects of IL-2 therapy. We also found that NO blocking agents alone can reduce tumor growth and spontaneous metastasis in this mammary tumor model in which tumor cells express eNOS. When combined with IL-2, NOS inhibitors improved IL-2 induced antitumor cytotoxicity, as well as tumor regression. Thus, NO blocking agents may be useful in treating tumors producing NO and serve as valuable adjuncts to IL-2 based therapies of cancer and infectious diseases.

Toxic side effects of systemic high dose IL-2 therapy, including capillary leakage, have recently forced investigators to seek alternate forms of IL-2 delivery, including gene therapy. These efforts have so far been less than promising in limited human trials. We propose that more research should be invested into combination therapies for achieving the dual benefit of amelioration of IL-2 toxicity and augmentation or the antitumor efficacy of systemic IL-2 therapy. A recent report [108] indicates that induction of oxygen-free-radicals may represent an additional arm of endothelial injury caused by IL-2 therapy, since treatment with dimethylthiourea (a scavenger of oxygen-free-radicals) attenuated IL-2 therapy-induced capillary leakage. We suggest that formation of peroxynitrite, a potent endotheliotoxic molecule, due to combination of NO with superoxide may be the strongest mediator of IL-2 induced capillary damage. It remains to be seen whether combination therapies designed to block both NO and superoxide can provide better means of controlling IL-2 toxicity and improving antitumor effects of IL-2.

Key unanswered questions

1. Can more specific iNOS inhibitors such as L-N^6-(1-Iminoethyl)-lysine hydrochloride (NIL) [109] or 1400W [110] given continuously in an osmotic minipump provide better protection from IL-2 induced mortality in mice than L-NAME?
2. How does IL-2 therapy-induced NO mediate the damage to endothelial cells: by direct NO-mediated

apoptosis, or endothelial injury caused by an over production of peroxynitrite, or both?
3. Will the combination of an iNOS inhibitor and an oxygen – free – radical – scavenger (such as dimethylthiourea) prove to be superior to either of these agents alone in preventing IL-2 induced capillary leakage and mortality?

Acknowledgements

Studies reported in this paper were supported by grants from the National Cancer Institute of Canada with the funds from the Canadian Cancer Society Inc., and sponsored in part by the Department of United States Army Grant DAMD 17-96-6096 to PKL. AO is the recipient of a postdoctoral fellowship from the Medical Research Council of Canada.

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Cancer and Metastasis Reviews **17**: 1–6, 1998.

Overview

Significance of nitric oxide in carcinogenesis, tumor progression and cancer therapy

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Key words: nitric oxide, nitric oxide synthase, carcinogenesis, tumor progression, metastasis, cancer therapy

It took nearly seven years to establish that 'endothelium derived relaxing factor (EDRF)', an elusive factor responsible for relaxation of vascular smooth muscle cells [1] was nothing other than nitric oxide (NO), a small gaseous molecule with significant bioactivity [2]. This discovery led to a major surge of research on the biology of NO, which has influenced nearly every discipline in biomedical sciences, and in 1992, the editors of Science annointed NO as the 'molecule of the year' [3]. NO has an unpaired electron and is thus a free radical capable of avid reaction with other molecules. This gives NO a very short half life (usually a matter of seconds) and a short range of bioactivity. It is a potent biological messenger in a variety of tissues, for a number of physiological functions such as vasodilation, inhibition of platelet aggregation within the microvasculature, regulation of neurotransmission and natural defense of the immune system. NO production by cells requires the presence of one or more of the three isoforms of NO synthases (NOS) recognized so far [4, 5]. Two of them (endothelial type NOS or eNOS; and neuronal type NOS or nNOS) are expressed constitutively and require the presence of intracellular Ca++ and calmodulin; the other isoform (inducible type NOS or iNOS, discovered initially in macrophages, hence also called mNOS) is usually induced in the body during inflammation by the presence of certain inflammatory cytokines and/or bacterial products. iNOS can function independently from cytosolic Ca++ and calmodulin because calmodulin is tightly bound to this isoform in a noncovalent manner, and trace levels of intracellular Ca++ can maintain its activity [5, 6]. Constitutive expression of the inducible isoform and induced activation of the constitutive isoforms have also been reported. Genes for all these isoforms have been cloned in numerous species [6, 7] and disrupted in mice to show that none of the disruptions was embryo-lethal, but each had selective pathological effects generally consistent with known biological functions of NO. For example, eNOS knockouts [8] are hypertensive, because of the vasorelaxant role of NO made by eNOS-bearing endothelial cells; iNOS knockout mice are susceptible to infection and show poor macrophage killer function against parasites and tumor cells [9] consistent with the recognized NO-mediated anti-bacterial, anti-parasitic and anti-tumor defense exerted by iNOS bearing macrophages; nNOS knockout mice show hypertrophic pyloric stenosis consistent with the inhibitory neurotransmitter role of NO in relaxation of pyloric sphincter muscles [10]. Male nNOS knockout mice also show aggressive sexual behaviour [11].

In spite of a rapid growth in the field of NO research which has attracted scientists from a variety of disciplines, and the recognition that NO may play key roles in a variety of pathological processes including cancer, the place of NO in cancer research remains at its infancy. Because of the recognition that NO is a free radical, significant research has been invested into the role of NO in mutagenesis and thus indirectly, carcinogenesis. More recent data suggest that NO exerts a major influence in tumor progression and cytokine therapy of cancer. A full understanding of the molecular biology of NO synthesis and its subsequent fate is essential in or-

der to appreciate the roles of NO in carcinogenesis, tumor progression and cancer therapy. The article by Geller and Billiar provides an excellent review of the topic, and sets the stage for the subsequent articles. This article demonstrates that NO biosynthesis can be regulated by a variety of mechanisms which influence the expression of NOS enzymes and their activity. They can be transcriptional or post-transcriptional (translational and post-translational). Species differences in some of the regulatory mechanisms, in particular, those accounting for poor inducibility of iNOS in human macrophages are illustrated. The authors also review mechanisms which turn off iNOS expression at the mRNA or protein levels.

The physiological functions of NO such as vasodilation, inhibition of platelet aggregation and regulation of neural activity have been shown to be dependent on NO-mediated increases in the intracellular levels of soluble guanylate cyclase and subsequently an increase in the cGMP levels [12, 13]. NO-mediated antibacterial or antiparasitic action of macrophages has been ascribed to the inhibition of enzymes essential for respiration; the antitumor (cytostatic and/or cytotoxic) function of macrophage-derived NO has been attributed to the inhibition of mitochondrial respiration as well as DNA-synthesis in the target cells [14]. The latter action is believed to be due to inhibition of ribonucleotide reductase [15], a rate limiting enzyme for DNA synthesis, as well as deamination of DNA bases [16].

Pathological functions of NO are attributed to NO reaction products. NO reacts with molecular oxygen, transition metals and superoxide resulting in intermediates which can cause cellular injury. In the presence of superoxide anion, NO is converted into peroxynitrite, a toxic molecule of high potency [17]. It has been recognized for some time that many of these intermediates can cause damage to DNA and thus be potentially carcinogenic in the long term. At what point does NO abandon its normal physiological role to become harmful for the body in the short or the long term? It would appear that constitutive production of NO for normal cellular functions is maintained steadily at a low level, so that production of toxic intermediates is minimized. On the other hand, induced NO production following inflammation, although helpful in the short term for normal body defense, may result in toxic intermediates capable of causing tissue damage and genotoxicity, and thus have potential carcinogenic effects. Felley-Bosco discusses how reactions with NO can give rise to genotoxic compounds within the body which may be potentially carcinogenic. She shows that compounds resulting from the reaction of NO with oxygen (N_2O_3 and $ONOO^-$) or superoxide (peroxynitrite) can damage cellular DNA directly or indirectly. Direct damage includes DNA base deamination following nitrosation of the amino groups in DNA bases, peroxynitrite-induced adduct formation and single strand breaks in DNA. Indirect damage can result from interaction of these compounds with other molecules such as amines, thiols and lipids, or an inhibition of DNA repair enzymes by these compounds. Mutagenic events, although presumed to be carcinogenic, have yet to be firmly linked with carcinogenicity at the tissue level.

It would appear that induced production of NO by macrophages and other cells in the body, or an aberrant production of NO in the tumor bearing host by tumor cells, endothelium of the tumor vasculature or macrophages within the tumors may act as a double-edged sword against the tumor or the host, depending on the circumstances. On one hand, NO can defend the host by arming host macrophages with antitumor activity [14]. Furthermore, excess NO production by tumor cells may be detrimental to their own survival due to NO-induced apoptosis [18]. On the other hand, significant experimental as well as clinical data suggest that tumor-derived NO is conducive to tumor progression and metastasis and thus detrimental to the host [19–24].

These apparently opposing roles of NO on the tumor-host balance may suggest that NO effects on tumor biology are hopelessly complex, so that one would never be able to exploit this knowledge to contain tumor growth or metastasis. This thought, although depressing at the outset, served as a major incentive and challenge for this guest editor to commission expert contributors who can adequately deal with both the antitumor and the tumor-facilitatory roles of NO so that the reader can form a

balance opinion. Upon reading these articles, the reader will be able to appreciate the fact that the data on the roles of NO on tumor biology are not conflicting after all, and that they provide important leads for therapeutic application of the knowledge under appropriate circumstances and open avenues for further experimentation.

To explore the possible antitumor function of NO, Albina has provided a succinct review of the role of NO in macrophage-mediated cytotoxicity and induction of apoptosis in the tumor cell target. It is shown that NO or NO-intermediates form an important but not exclusive component of the machinery employed by macrophages in killing tumor targets; that apoptosis is a key but not exclusive mechanism in the NO-mediated cell death; that the apoptotic event can result from numerous mechanisms including oxidative injury; that NO induction may eventually be counterproductive for this macrophage function because of NO-mediated effects on the macrophages themselves, such as suppression of cytotoxic function, and eventual suicide by apoptosis. It is also shown that tumor cells can develop resistance to NO-mediated killing. Finally, the author cautions the readers that the bulk of the data supporting the antitumor role of NO induced in macrophages is derived from nonhuman species (mostly rodents) and that such evidence is weak in the case of human macrophages which show poor induction of NOS activity. Indeed, it is suggested that low levels of NO production by human macrophages may have facilitating effects on tumor growth (a view supported by the data presented in the article by Thomsen and Miles).

As pointed out earlier, high levels of NO when induced in certain cells can cause cytostasis and apoptosis. Can this phenomenon be exploited as a therapeutic manoever to reduce tumor cell survival and induce tumor cell death? The article by Xie and Fidler demonstrates that this can be achieved in certain tumors. Using murine melanoma cell lines as vehicles for genetically engineered overexpression of the iNOS gene, they show that iNOS overexpressing cells have poor growth and survival *in vitro* as well as *in vivo* in the absence of NOS inhibitors because of NO-induced apoptosis and these cells lose their ability for metastasis *in vivo*. They also show that multiple systemic administration of lipopeptide-containing liposomes along with interferon (IFN)-γ was highly effective in causing regression of established hepatic metastases of a murine reticulum cell sarcoma by inducing iNOS expression in tumor cells *in vivo*. These studies illustrate the potential of gene therapy for certain tumors or NOS-inducing biologic therapy of NOS inducible tumors which are susceptible to NO-mediated apoptosis. In apparent contradiction of these results, Jenkins *et al.* [23] reported that introduction of functional iNOS into a human colonic adenocarcinoma cell line led to increased growth and vascularity of the tumors when transplanted in nude mice (see the article by Thomsen and Miles for further details). A close scrutiny of the results reported by the two groups suggests that the data may not be contradictory after all. In the case of the murine melanoma, iNOS overexpression led to much higher levels of NO production by tumor cells than in the case of the human colonic adenocarcinoma line, so that in the former case this was apoptosis-inducing, whereas in the latter case it was not. It is also likely that tumor cells may vary in their susceptibility to NO-mediated injury. It has been suggested that the cytostatic effects of NO are observed in those tumor cells which express functional wild-type p53 because of NO-mediated accumulation of p53 protein, whereas tumor cells with missing or mutated p53 exhibit resistance ot NO-mediated injury and promotion of tumor growth in the presence of endogenous NO [25] (see article by Lala and Orucevic for further details). There may be other genetic determinants which dictate the role of NO in tumor biology. Thus, it is conceivable that, *in vivo*, very high NO-producing tumor cells which are susceptible to NO-mediated killing would die and thus be selected against; only cells capable of surviving (either owing to lower levels of NO production or resisting NO-mediated killing) will have a growth advantage and may further utilize NO as a tool for promotion of aggressive behavior by a variety of possible mechanisms as listed below.

Now let us turn to the possible facilitatory role of NO in the progression of certain tumors. Data on human tumor models are reviewed by Thomsen and Miles, and experimental tumor models by Lala

and Orucevic. A positive correlation of NOS expression with tumor progression has been reported in numerous human tumor models: tumors of the central nervous system [20], gynecological cancer [19] and breast cancer [21], models which are discussed in the article by Thomsen and Miles. A similar correlation is also noted in a murine mammary tumor model (see the article by Lala and Orucevic).

Thomsen and Miles focus particularly on their findings of the association of iNOS expression by stromal (macrophage and endothelial) cells within the tumor with tumor grade in human breast and gastric cancer specimens. They suggest that cytokines-chemokines in the tumor microenvironment may provide appropriate signals for recruitment and activation of macrophages capable of steady low level NO production, which facilitates tumor progression. In support of this hypothesis, they show that tumor-infiltrating macrophages express CD23, the low affinity receptor for IgE reported to trigger iNOS induction after receptor ligand interaction. They also show that administration of a selective iNOS inhibitor, 1400W, limited the *in vivo* growth of an iNOS-transduced human colonic adenocarcinoma in nude mice and an iNOS-expressing EMT6 mammary adenocarcinoma in syngeneic mice.

What are the underlying mechanisms that may allow NO to promote tumor progression/metastasis? A number of possibilities are discussed in different articles in this issue. In the article by Fukumura and Jain, the authors briefly review the angiogenic role of NO and provide data from their own laboratory on the facilitatory role of NO in tumor blood flow within the microvasculature. They describe an elegant skin window model to measure hemodynamics and microcirculation in superficial tumors with the aid of videomicroscopy. It is shown that endogenous NO (derived from tumor vascular endothelium and/or tumor cells) promotes and/or maintains tumor blood flow via dilation of arteriolar vessels, decreases leukocyte-endothelial adhesive interactions and increases vascular permeability. Exogenous NO stimulated tumor blood flow and reduced vessel tone. NO production and vascular responses were tumor-dependent. Thus. NO-mediated promotion of microcirculation in certain tumors is a key event that facilitates tumor growth by ensuring adequate nutrient supply through the tumor vasculature. This is an important aspect of tumor biology which can be exploited for altering tumor hemodynamics and thus improving the delivery of drugs, gene vectors and effector cells to target cells of the solid tumor.

There is a growing body of evidence to indicate that tumor-derived NO promotes tumor angiogenesis as well invasiveness of certain human (see the article by Thomsen and Miles) and animal (in the article by Orucevic and Lala) tumors. The angiogenic role of NO was shown in iNOS transduced human colonic adenocarcinoma grown in nude mice (in the article by Thomsen and Miles), and eNOS expressing mammary adenocarcinomas in C3H/HeJ mice (in the article by Lala and Orucevic). In the latter model, it was further shown that tumor cell invasiveness required for local spread as well as metastasis was enhanced by tumor-derived NO. The increased invasiveness resulted from alterations in the balance in the production of matrix metalloproteases (MMPs) and their natural inhibitors (TIMPs). Consistent with these findings, Thomsen and Miles, in their article, show that treatment of hosts with the iNOS inhibitor 1400W reduced local tissue invasion by iNOS expressing EMT6 tumor cells.

Cytokine-based immunotherapies have recently opened important new avenues in treating certain human cancers. Of these, high dose IL-2 therapy provided the strongest hope in the mid 80's and early 90's in treating renal cell carcinoma and melanoma patients. This was, however, marred by a major side effect termed 'Capillary Leak Syndrome' characterized by severe hypotension and fluid leakage into tissue spaces. It had been shown that lymphokine (IL-2) activated killer (LAK) cells can inflict direct damage to endothelial cells *in vitro* [26] which could, at least in part, explain the IL-2 induced capillary leakage *in vitro*. Since LAK cells themselves represent the most important therapeutic arm of IL-2 based therapies in causing cancer regression, it appeared that one could not mitigate the capillary leakage without compromising the anticancer effects of IL-2. Fortunately, however, another key player, NO, has recently been recog-

nized as the prime mediator of the hypotensive as well as the endothelio-toxic effects of IL-2 therapy. Shahidi and Kilbourne review the roles of cytokines *in vitro* and *in vivo* in inducing iNOS and the application of NOS inhibitors, NO scavangers or tetrahydrobiopterin antagonists for ameliorating IL-2 therapy-induced hypotension. In their own series of studies, utilizing dogs as well as a limited number of human subjects receiving high dose IL-2 therapy, they show that the induction of NO could account for the major hypotensive effects of IL-2 therapy, and that combined therapy with NOS inhibitors could ameliorate IL-2 therapy-induced hypotension without compromising its therapeutic efficacy. A clinical study in metastatic melanoma-bearing patients is currently in progress at the National Institutes of Health, USA.

A series of studies in mice by Orucevic and her colleagues (see article by Orucevic and Lala) revealed that IL-2 therapy-induced NO also played a major role in causing capillary damage. They show that a combination of NO inhibitors (L-NAME or NMMA given chronically by the oral route) with IL-2 therapy provided a dual benefit: amelioration of capillary leakage, and improvement of therapeutic (antitumor/antimetastatic) effects of IL-2 therapy. The latter was, at least in part, shown to be due to an improvement of antitumor killer function of LAK cells generated *in vivo*, indicating that NO induced by IL-2 therapy interfered with optimal LAK cell activation. Thus it appears that high levels of NO induction can have general suppressive effects on the antitumor effector cells: LAK cells (in the above article) as well as killer macrophages (in the article by Albina).

The current issue of this journal should stimulate further research on the role of NO in tumor biology and an exploitation of the knowledge for cancer therapy. Additional basic research is needed to identify the circumstances for the tumor-inhibitory and facilitatory roles of NO and also the possible tumor-markers which may predict such roles. Several areas of translational research have potential in the near future: 1) modulation of tumor hemodynamics to improve the delivery of therapeutic agents to solid tumors; 2) therapy with iNOS inducing agents in iNOS inducible cancers, which are susceptible to NO-mediated cytotoxicity; or the use of iNOS transduced tumor cells as a vaccine, similar to the cytokine gene-transduced tumor cells currently being tested; 3) use of NOS inhibitors as a therapeutic adjunct in tumors which exhibit NO-induced tumor progression; 4) a revival of systemic IL-2 therapy along with NOS inhibitors. This combination therapy has potential for treating certain cancers as well as septic shock. Current availability of relatively nontoxic and selective inhibitors of iNOS such as aminoguanidine and 1400 W makes this a realistic goal to achieve. Finally, a good knowledge of the intermediary metabolism of NO should provide newer leads into the prevention of NO-induced tissue damage and genotoxicity following inflammatory conditions.

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NITRIC OXIDE PRODUCTION BY MURINE MAMMARY ADENOCARCINOMA CELLS PROMOTES TUMOR CELL INVASIVENESS[1]

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keywords: nitric oxide, mammary cancer, invasiveness, MMP, TIMP

[1] This study was supported by funds from the Department of the United States Army grant DAMD 17-96-6096 to PKL, funds from the NIH grant # GM44100 to TRB and a Postdoctoral Fellowship from Medical Research Council of Canada to AO.

Abstract

The role of nitric oxide (NO) in tumor biology remains controversial and poorly understood. While a few reports indicate that the presence of NO in tumor cells or their microenvironment is detrimental for tumor cell survival and consequently their metastatic ability, a large body of data suggest that NO promotes tumor progression. The purpose of this study was to identify the source of NO in the spontaneously metastasizing C3-L5 murine mammary adenocarcinoma model, the role of tumor - derived NO in tumor cell invasiveness, and the mechanisms underlying the invasion-stimulating effects of tumor derived NO. The source of NO was established by immunocytochemical localization of NO synthase (NOS) enzymes in C3-L5 cells in vitro and transplanted tumors in vivo. An in vitro transwell matrigel invasion assay was used to test invasiveness of C3-L5 cells in the presence or absence of NO blocking agents or iNOS inducers (IFN γ and LPS). The mechanisms leading to the observed invasion-stimulating effects of tumor derived NO were examined by measuring mRNA expression of matrix metalloproteinases (MMP)-2 and 9 and tissue inhibitors of metalloproteinases (TIMP) 1, 2 and 3 in C3-L5 cells under various experimental conditions. Results revealed that C3-L5 cells expressed high level of eNOS protein in vitro, as well as in vivo in primary and metastatic tumors. The cells also expressed iNOS mRNA and protein following culture with IFN γ and LPS. Constitutive NO produced by C3-L5 cells promoted tumor cell invasiveness in vitro by down-regulating TIMP 2 and TIMP 3; in addition, there was an upregulation of MMP-2, when extra NO was induced by IFN γ and LPS. In conclusion. NO produced by C3-L5 cells promoted tumor cell invasiveness by altering the balance between MMP-2 and its inhibitors TIMP-2 and 3. Thus, our earlier observations of antitumor and antimetastatic effects of NO inhibitors in vivo in this tumor model can be explained, at least in part, by reduced tumor cell invasiveness.

Introduction

Nitric oxide (NO)[2] is synthesized in mammalian cells from amino acid L-arginine by a family of enzymes. the nitric oxide synthases (NOS) (1,2). This molecule plays a key role in many physiological as well as pathological processes, including inflammation and neoplasia. Numerous clinical and experimental studies indicate a contributory role of NO in tumor progression. The level of NOS protein (3,4), as well as NOS activity (3) has been positively correlated with the degree of malignancy in a number of human cancers, including human gynaecological (ovarian, uterine) cancers (3), central nervous system tumors (4) as well as breast cancer (5). The high NOS activity has been explained by the presence of constitutive form(s) in tumor cells (3,4) and tumor endothelial cells (4), or the expression of the inducible form in tumor endothelial cells (4) or tumor associated macrophages (5). Expression of iNOS in the tumor neovasculature has also been reported in experimental tumors (6,7). Furthermore, several lines of direct evidence exist for a facilitatory role of NO on tumor progression. (a) In a rat colonic adenocarcinoma model, treatment with N^G-Nitro-L-Arginine methyl ester (L-NAME), an NO synthase inhibitor, was shown to reduce NO production as well as tumor growth (7). (b) Similarly, antitumor as well as antimetastatic effects of two inhibitors of NO, N^G-Methyl-L-Arginine (NMMA) and L-NAME, have been observed in our laboratory, using a mouse mammary adenocarcinoma model (8,9). (c) Recently, Edwards *et al.* (10) observed that NO induced by lypopolysaccharide (LPS) and interferon (IFN)-γ in EMT-6 murine mammary carcinoma cells stimulated tumor growth and metastasis in vivo, in spite of NO induced inhibition of cell growth in vitro. (d) Finally, numerous human colon cancer cell lines are found to express NOS activity (11), and engineered overexpression of iNOS in a human

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[2] Abbreviations: NO nitric oxide, NOS nitric oxide synthase; eNOS endothelial NOS; iNOS inducible NOS; IFN interferon; L-NAME NG-Nitro-L-Arginine methyl ester; LPS lipopolysaccharide, MMP matrix metalloprotease; NMMA NG-Methyl-L-Arginine; TIMP tissue inhibitor of metalloprotease.

colonic adenocarcinoma line was found to increase tumor growth and vascularity when transplanted in nude mice (12).

In apparent contradiction to the above reports, the presence of NAD(P)H diaphorase, NOS enzymes and NOS activity in the human colonic mucosa, polyps and carcinomas, appeared to be inversely related to colonic tumor progression (13,14). Similarly, high NOS activity was inversely correlated to tumor growth and metastasis in a murine melanoma model; an engineered overexpression of iNOS in the melanoma cells reduced tumorigenicity, because of NO mediated tumor cell apoptosis (15). These reported discrepancies may be explained by dual role of NO on tumor growth. Whereas very high NO producing tumor cell clones may delete themselves by apoptosis, NO may facilitate in vivo growth of surviving clones by numerous mechanisms, including promotion of neoangiogenesis, increased tumor blood flow, increased invasiveness, or by inhibition of apoptosis. While some evidence exist for contributory role of NO in promotion of neoangiogenesis (6,16) and tumor blood flow (17), the possible role of tumor derived NO on invasiveness has not been explored.

The mechanisms by which NO promotes growth or metastasis of murine mammary adenocarcinoma remained undefined (8,9). The objectives of the present study were to identify the source of NO in this spontaneously metastasizing C3-L5 mammary adenocarcinoma model, its role on tumor cell invasiveness and the mechanisms underlying the observed invasion-stimulating effects of NO.

Materials and methods

Tumor cell line and culture conditions

C3-L5 mammary adenocarcinoma cell line was selected and maintained in P.K. Lala's laboratory from its parent C3 line, by 5 cycles of in vivo selection for spontaneous lung micrometastases following s.c. transplantation in C3H/HeJ mice (18). C3 line had been clonally derived from a primary transplantable tumor T58. T58 was grown from a spontaneous mammary tumor in a C3H/HeJ retired breeder female mouse, which also

exhibited spontaneous lung metastases (19). While the strong ability for spontaneous metastasis to the lungs originally exhibited by C3 cells declined over the years of in vitro passages, C3-L5 line has maintained its ability to metastasize spontaneously to the lungs. After thawing, C3-L5 cells were grown in RPMI 1640 medium (Gibco BRL, Burlington, ON) with 1% antibiotics (Mediatech, Washington, DC), supplemented with 10% fetal calf serum (Gibco BRL, Burlington, ON) in 75 cm^2 flasks (Becton Dickinson and Co., Lincoln Park, NJ) until confluent. Antibiotics contained 5,000 I.U./ml of Penicillin and 5,000 mcg/ml of Streptomycin. All experiments were done with cells passaged 3 - 5 times after thawing.

In experiments designed to block NO synthesis, L-NAME and NMMA (Sigma Chemical Company, St. Louis, MO) were added to the medium at concentrations of 0.01 - 1 mM; in some experiments, L-NAME was used at a concentration of 1 mg/ml of medium (equivalent of 3 mM).

In experiments designed to induce iNOS and thereby increase NO production, a combination of recombinant murine IFN γ (500-100 U/ml) and LPS (10 μg/ml) was added to the culture medium. IFN γ (lot FC2B11) was obtained from Gibco (Burlington, ON), reconstituted with sterile water in aliquots of 10,000 U/100 μl and stored at -70°C until used for assays. LPS powder was obtained from Sigma (St.Louis, MO), stored at 4°C, dissolved with complete medium on the day of assay and sterilized by filtration (0.2 μm filter pore size, Nalgene syringe filters, Nalgene Co., Rochester, New York).

Experimental procedures

Immunocytochemical localization of NOS enzymes in C3-L5 cells and primary tumor tissue

C3-L5 cells were grown for 24 h on chamber slides, either in complete medium alone or in medium containing 500 U/ml of IFN γ or 10 μg/ml of LPS or combination of IFN γ + LPS in a humidified incubator (37°C, 5% CO_2 atmosphere). Slides were briefly washed with phosphate buffered saline (PBS), fixed in 10% buffered formalin, and

permeabilized with 0.25% Triton X-100 in PBS. After washing (3x5 min PBS), 10% normal goat serum was added to the slides as blocking serum. Slides were then subjected to following treatments followed by washes: Mouse monoclonal antibody against macrophage iNOS and endothelial NOS (eNOS) (Transduction Laboratories, Lexington, KY, 1:50 dilution) was used for identification of iNOS and eNOS expression in C3-L5 cells, respectively. Slides with secondary goat anti-mouse biotinylated antibody (Dimension Laboratories, Mississuaga, ON, 1:200 dilution) were incubated 30 min, and followed with ABC complex (1h) and DAB chromogen. Slides were counterstained with hematoxylin, and NOS expression identified by positive brown staining from DAB chromogen under light microscope. Negative controls were provided by omission of primary antibodies, or a substitution of the primary antibodies with equivalent concentration of normal mouse Ig.

Samples of primary tumors grown in C3H/HeJ mice for 24 days, following subcutaneous transplantation of 2.5 x 10^4 C3-L5 cells, as well as their spontaneous lung metastases were fixed in 10% buffered formalin, paraffin embedded, and cut at 4 μm thick sections. After deparaffinization, and blocking of endogenous peroxidase activity (3% H_2O_2 in absolute methanol), sections were permeabilized with 0.25% Triton X-100 in PBS. Ten percent normal horse serum or normal goat serum was added to the slides as blocking sera, followed by mouse monoclonal primary antibody against iNOS or rabbit polyclonal antibody against eNOS (Affinity Bioreagents, Neshanic Station, NJ, 1:200 dilution) and incubated overnight. Secondary horse anti-mouse biotinylated antibody was added after primary monoclonal antibody, and goat anti-rabbit biotinylated antibody after primary polyclonal antibody treatment. ABC complex and DAB were added as described above, and sections were counterstained with hematoxylin. Negative controls were provided by omission or substitution of primary antibodies with normal mouse or rabbit Igs at equivalent concentrations.

Matrigel invasion by C3-L5 cells

An in vitro transwell matrigel invasion assay (20,21) was used to test invasiveness of C3-L5 cells in the presence or absence of 0.01, 0.1, 1 and 3 mM NO blocking agent L-NAME or NMMA or iNOS inducers, IFN γ (500-1000 U/ml) + LPS (10 μg/ml), in the presence or absence of L-NAME. Some wells contained excess L-arginine (5 times the concentration of NOS inhibitors, used as specificity control to abrogate the effects of NO inhibitors). In this assay, tumor cells were prelabelled with ^{3}HTdR for 24 h, and then added to the invasion chamber of the transwell containing a matrigel (reconstituted basement membrane, Collaborative Research, Inc.)-coated millipore membrane. Percentage of labelled cells penetrating the matrigel-millipore membrane were scored as percent radioactivity appearing in the lower well and bottom of the millipore membrane, as a function of time (1-3 days). All assays were done in triplicate.

Measurement of NO production in the media from C3-L5 cells

C3-L5 cells were grown in 24 well plates (10^6 cells/800 μl media/well) either in the medium alone, L-NAME alone (1mg/ml) or in the presence of IFN γ (500 - 1000 U/ml) + LPS (10 μg/ml) + L-NAME. Culture media from wells were collected after 24h of the incubation period, and kept frozen at -20°C, until assayed for NO_2^- levels. Griess reagent (22) was used for measurement of NO_2^-.

Analysis of mRNA expression for matrix metalloproteases (MMP)-2 and 9 and tissue inhibitors of metalloproteases (TIMP) 1, 2 and 3 in C3-L5 cells.

C3-L5 cells were grown for 24h on 10 cm^2 tissue culture dishes either in RPMI medium with 1% bovine serum albumin or in the medium with NMMA or IFNγ + LPS ± NMMA. Total RNA was isolated by standard methods using RNAzol B (Biotec Laboratories, Houston Texas) (23). Twenty μg of total RNA from each sample was electrophoresed on a 1% agarose gel containing 3% formaldehyde prior to transfer to Gene Screen membrane (DuPont - New England Nuclear, Boston, MA) and UV autocrosslinked (UV Stratalinker 1800, Stratagene, La Jolla, CA). cDNA probes for TIMP 1, 2 and 3 were obtained from Dr. Rama Khokha (Ontario Cancer Institute,

Toronto, Canada) and murine MMP-2 (72 kD collagenase) and MMP-9 (92 kD collagenase) probes were obtained from Dr. Dylan Edwards (Calgary, Alberta, Canada). cDNA probe for murine iNOS was obtained from Dr. Charles Lowenstain (John Hopkins, Baltimore, MD). These cDNA probes were labeled with [^{32}P]dCTP by random priming. Hybridizations were carried out overnight at 43^{o}C and hybridized filters were washed at 53^{o}C, following methods from published protocols (24,25). Autoradiography was performed at -70^{o}C in the presence of intensifying screen. Northern blot membranes were stripped for rehybridization with 18s rRNA utilized as loading controls. Relative mRNA levels were quantitated by PhosphorImager scanning using the ImageQuant software (Molecular Dynamics, Inc. Sunnyvale, CA).

Results

Expression of immunoreactive eNOS protein by C3-L5 cells in vitro and in vivo

Immunocytochemical staining for eNOS enzyme revealed that C3-L5 mammary carcinoma cells constitutively expressed high level of eNOS protein in vitro (Figure 1.A).

Subcutaneous tumors grown in C3H/HeJ mice for 24 d after transplantation of 2.5 x 10^4 C3-L5 cells, as well as their spontaneous metastatic counterparts (lungs) expressed eNOS protein (Figure 2 A and C). eNOS protein was present, on the average, in approximately 80% of tumor cells at the primary tumor site, and in about 40% of the tumor cells in the lung metastatic nodules.

Expression of iNOS in C3-L5 cells in vitro

C3-L5 cells did not express iNOS mRNA under native conditions, however, iNOS mRNA was induced by 24 h stimulation with IFNγ + LPS (500 U/ml and 10 μg/ml, respectively). This induction was upregulated by treatment of cells with NMMA (Figure 3.A). IFN γ and LPS treatment induced significant increase in NO production, measured as NO_2^- levels in the medium. Additional presence of NMMA or L-NAME significantly reduced NO_2^- levels in the medium, but not to the control levels (Figure 3.B.) Expression of immunoreactive iNOS protein after induction with combination of IFN γ+ LPS

(Figure 1.C.) was correlated with mRNA expression. IFN γ alone or LPS alone did not induce iNOS mRNA or protein in C3-L5 cells (data not presented).

Invasiveness of C3-L5 cells in vitro

C3-L5 cells, on their own, exhibited significant invasiveness in 3d matrigel invasion assay, as indicated by an invasion index of 50 ± 5%. This value is normalized to 100% in Figure 4 to test the effects of NMMA. NMMA at 0.25-1 mM concentrations reduced the invasiveness of C3-L5 cells. Addition of excess L-arginine abrogated the NMMA effects, indicating the specificity of NMMA action (Figure 4).

Another NOS inhibitor. L-NAME, at concentrations of 0.01 - 1 mM significantly ($p<0.05$) reduced invasiveness of C3-L5 cells in a 3 d matrigel invasion assay, as was previously noted with NMMA. (Figure 5).

Combination of IFN γ and LPS (500 U/ml and 10 μg/ml, respectively) significantly ($P<0.05$) stimulated invasiveness of C3-L5 cells in 3 d matrigel invasion assay (Figure 6). L-NAME (1 mg/ml) reduced this invasiveness only minimally (Figure 6 - not significant.). A combination of IFN γ and LPS at higher concentrations (1000 U/ml and 10 μg/ml, respectively), also significantly stimulated invasiveness of C3-L5 cells in both 1 d and 3 d matrigel invasion assays in separate experiments (data not presented).

mRNA expression of MMPs and TIMPs in C3-L5 cells treated with IFNγ, LPS and NMMA

IFNγ and LPS treatment upregulated MMP-2 (72kD collagenase) mRNA expression in C3-L5 cells, and addition of NMMA to this treatment restrained the mRNA expression to the control level. NMMA alone did not have any effect on MMP-2 mRNA expression (Figure 7. A.). C3-L5 cells did not express MMP-9 (92 kD collagenase) mRNA under any conditions (data not shown).

IFNγ and LPS treatment with or without NMMA, or NMMA treatment alone did not significantly influence TIMP 1 mRNA expression (Figure 7. B.).

IFNγ and LPS treatment caused a minor down-regulation of TIMP-2 mRNA (Figure 7. C.), and a strong down-regulation of TIMP-3 mRNA (Figure 7.D.) expression Addition of NMMA to LPS and IFNγ only partially restored TIMP-3 expression (Figure 7. D.). NMMA treatment alone resulted in a small upregulation of both TIMP-2 and TIMP-3 mRNA expression (Figure 7.C. and D.).

Discussion

Results from the present study revealed that in vitro propagated C3-L5 mammary adenocarcinoma cells expressed eNOS protein and in addition, were stimulated to express iNOS protein, when grown in the presence of IFN γ + LPS. Tumor cells grown in vivo expressed eNOS, but not iNOS protein, both at the primary site, as well as the sites of spontaneous lung metastasis. These cells exhibited a strong ability to invade matrigel, and their invasiveness was reduced in the presence of NO blocking agents (NMMA and L-NAME). This was paralleled with upregulation of TIMP-2 and TIMP-3 mRNA expression. The anti-invasive effects were abrogated in the presence of excess L-arginine, attesting to fact that the effects were due to an inhibition of NO synthesis. Finally, invasiveness of C3-L5 cells was stimulated in the presence of iNOS inducing agents IFNγ and LPS, with a concomitant increase in NO production in vitro. This was paralleled with upregulation of MMP-2 mRNA and a down-regulation of TIMP-2 and TIMP-3 mRNA. In this case, addition of L-NAME failed to abrogate the invasiveness significantly, but did however, restore the 72 kD collagenase expression to the control level and only partially restored TIMP-3 expression. Taken together, these results demonstrated that NO production by C3-L5 cells promoted tumor cell invasiveness by altering the balance between the expression of MMP-2 and its inhibitors TIMP 2 and TIMP 3.

We have previously reported (8,9) that treatment of C3H/HeJ mice bearing C3-L5 mammary adenocarcinoma transplants, with NOS inhibitors NMMA and L-NAME, had significant anti-tumor and anti-metastatic effects. Growth retarding effects of L-NAME

were also observed in a rat colonic adenocarcinoma model (7), indicating that NO had a promoting role on tumor progression in these tumor models. Indeed, high NOS activity has been positively correlated to the progression of tumors of the human reproductive tract (3), central nervous system (4) and the mammary gland (5). In concurrence with these observations, C3-L5 mammary carcinoma cells, used in the present study, are found to express high levels of eNOS protein in vitro, as well as in vivo in primary and metastatic tumors. They also expressed iNOS protein following culture with IFN γ and LPS. These findings attest to the NO producing ability of these cells under constitutive conditions, which may be enhanced under inductive circumstances.

Multiple mechanisms may be postulated for the role of NO produced by tumor cells or host-derived cells in the promotion of tumor growth or metastases. Because of its vasodilatory function (26), NO may promote the blood flow through the tumor vasculature and thus indirectly promote tumor cell nourishment. This hypothesis was supported by the temporal relationship of the reduction in tumor growth to L-NAME therapy in a rat adenocarcinoma model (7). NO has been shown to have a stimulatory effect on angiogenesis in vitro (27), as well as in vivo, utilizing a rabbit cornea model (16), or a model of healing gastric ulcer (28). Angiogenesis-promoting role of NO was supported in a tumor model by the demonstration of increased vascularity of transplants of human colonic adenocarcinoma cells in nude mice, when these cells were engineered to over-express mouse iNOS gene (12). Furthermore, it is hypothesized that during the vascular stage of the growth of malignant melanoma, NO synthesis is elevated in order to maintain a vasodilator tone in and around the tumor (29). Our preliminary data (reviewed in 30) of a reduction in the C3L5 tumor induced angiogenesis, when the mice were subjected to NMMA therapy, also support this hypothesis.

Another possible mechanism, a direct NO-mediated stimulation of tumor cell proliferation, has been excluded in the C3-L5 tumor model. Treatment of C3-L5 cells in vitro with NMMA had no effect on ^{3}HTdR uptake by these cells (Orucevic and Lala,

unpublished). Finally, as demonstrated in this study, NO production by C3-L5 cells promoted tumor cell invasiveness, and this mechanism may explain, at least in part, the observed reduction of primary tumor growth, as well as spontaneous lung metastasis following NMMA or L-NAME therapy (8,9).

The mechanisms underlying the invasion-stimulating effects of NO in our tumor model appear to be in part due to up-regulation of MMP-2 under inductive conditions. Upregulation of TIMP-2 and TIMP-3 in the presence of NOS inhibitors alone indicated that the invasion-stimulating effects of endogenous NO are, at least in part, mediated by a downregulation of these protease inhibitors. Earlier studies have shown that NO promotes degradation of articular cartilages by stimulating certain MMPs (collagenase and stromelysin) in human, bovine or rabbit chondrocytes (31,32). In addition, it has been reported that NO generation ameliorates the tubulointerstitial fibrosis of obstructive nephropathy by reducing interstitial collagen IV and α-smooth muscle actin (33). It has also been shown that TIMP-1 can be inactivated by peroxynitrite, which is formed rapidly from NO and O_2^- in conditions such as inflammation and ishaemic reperfusion (34). Furthermore, NO has been shown to upregulate urokinase type plasminogen activator (uPA) in endothelial cells of postcapillary venules during the process of NO-mediated stimulation of angiogenesis (35). Since uPA converts plasminogen to plasmin, which can activate numerous MMPs, this may represent another pathway of NO-mediated stimulation of matrix degradation.

In apparent contrast to the above studies, an engineered overexpression of iNOS in K1735 murine melanoma cells leading to decreased tumor cell survival and tumorigenicity (15), has been reported to be associated with a downregulation of MMP-2 (36) owing to the down-regulation of its promoter activity. Furthermore, additional biological roles of TIMP-1, TIMP-2 and TIMP-3 other than protease inhibitors have been proposed. These molecules were reported to act as a growth promoting or inhibiting proteins (37-42) depending on the cell line. Thus, further studies are needed to evaluate

the biological consequences of NO mediated regulation of MMPs and TIMPs in other tumor cell systems. It is also possible that the genetic make-up of tumor cells may influence the biological role of NO in tumor progression (30,43.44).

In summary, NO mediated promotion of tumor cell invasiveness resulting from an altered balance between MMP-2 and its inhibitors, can partly account for the therapeutic benefits of therapy with NOS inhibitors in the C3-L5 mammary adenocarcinoma model.

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Figure legend

Figure 1. Immunoperoxidase staining of C3-L5 cells in culture for NOS enzymes

A - eNOS labelling is present in 90-95% of cells.

B - corresponding negative control (omission of primary antibody).

C - iNOS labelling is present in 15-20% of cells after induction with IFN γ + LPS.

D - corresponding negative control (omission of primary antibody). C and D were counterstained with hematoxylin.

Figure 2. Immunocytochemical localization of eNOS in primary tumors and their metastatic lung nodules, 24 days after sc.transplantation of C3-L5 cells into C3H/HeJ mice.

eNOS is present in approximately 80 % of tumor cells at the primary site (a) and approximately 40 % of tumor cells in a lung metastasis (c). (b) and (d) are corresponding negative control (omission of primary antibody). Sections were counterstained with hematoxylin.

Figure 3. iNOS induction and NO production following treatment of C3-L5 cells with IFNγ and LPS

Figure 3. A. mRNA expression of iNOS.

iNOS was not expressed by C3-L5 cells under native condition, but was induced by IFN γ and LPS. This induction was upregulated by treatment of cells with NMMA.

Figure 3. B. NO_2^- levels in the medium of C3-L5 cells after 24h treatment with IFNγ + LPS ± NMMA or L-NAME.

IFN γ and LPS treatment induced significant increase in NO production. Addition of NMMA or L-NAME significantly reduced NO_2^- levels in the medium, but not to the control levels.

Figure 4. Matrigel invasion by C3-L5 cells treated with different concentrations of NMMA (± L-arginine)

NMMA at concentrations 0.25 - 1 mM reduced ($p<0.05$) tumor cell invasiveness. Five times excess of L-arginine abrogated the NMMA effects indicating the specificity of NMMA action.

Figure 5. Matrigel invasion by C3-L5 cells treated with different concentrations of NMMA and L-NAME.

Both agents at all concentrations (0.01 to 1 mM) reduced ($p<0.05$) tumor cell invasiveness.

Figure 6. Matrigel invasion by C3-L5 cells treated with IFN γ (500 U/ml) + LPS (50 ng/ml).

* significantly ($p<0.05$) different from control.

IFN γ + LPS significantly ($p<0.05$) stimulated invasiveness of C3-L5 cells in 3 d matrigel invasion assay. Reduction of IFN γ +LPS induced invasiveness with addition of L-NAME (1 mg/ml) was not significant.

Figure 7. A. mRNA expression of MMP-2 (72 kD collagenase).

IFN γ and LPS treatment upregulated MMP-2 mRNA expression in C3-L5 cells, and addition of NMMA to this treatment restrained the collagenase mRNA expression to the control level. NMMA alone did not have any effect on MMP-2 expression.

Figure 7. B. mRNA expression of TIMP 1.

IFN γ and LPS treatment with or without NMMA, or NMMA treatment alone did not significantly influence TIMP 1 mRNA expression.

Figure 7. C. mRNA expression of TIMP 2.

IFN γ and LPS treatment down-regulated TIMP 2 mRNA expression. Addition of NMMA to LPS and IFN γ did not restore TIMP 2 expression. NMMA treatment alone, however, upregulated the expression of TIMP 2.

Figure 7. D. mRNA expression of TIMP 3

IFN γ and LPS treatment down-regulated TIMP 3 mRNA expression. Addition of NMMA to LPS and IFN γ partially restored TIMP 3 expression. NMMA treatment alone, however, upregulated the expression of TIMP 3.

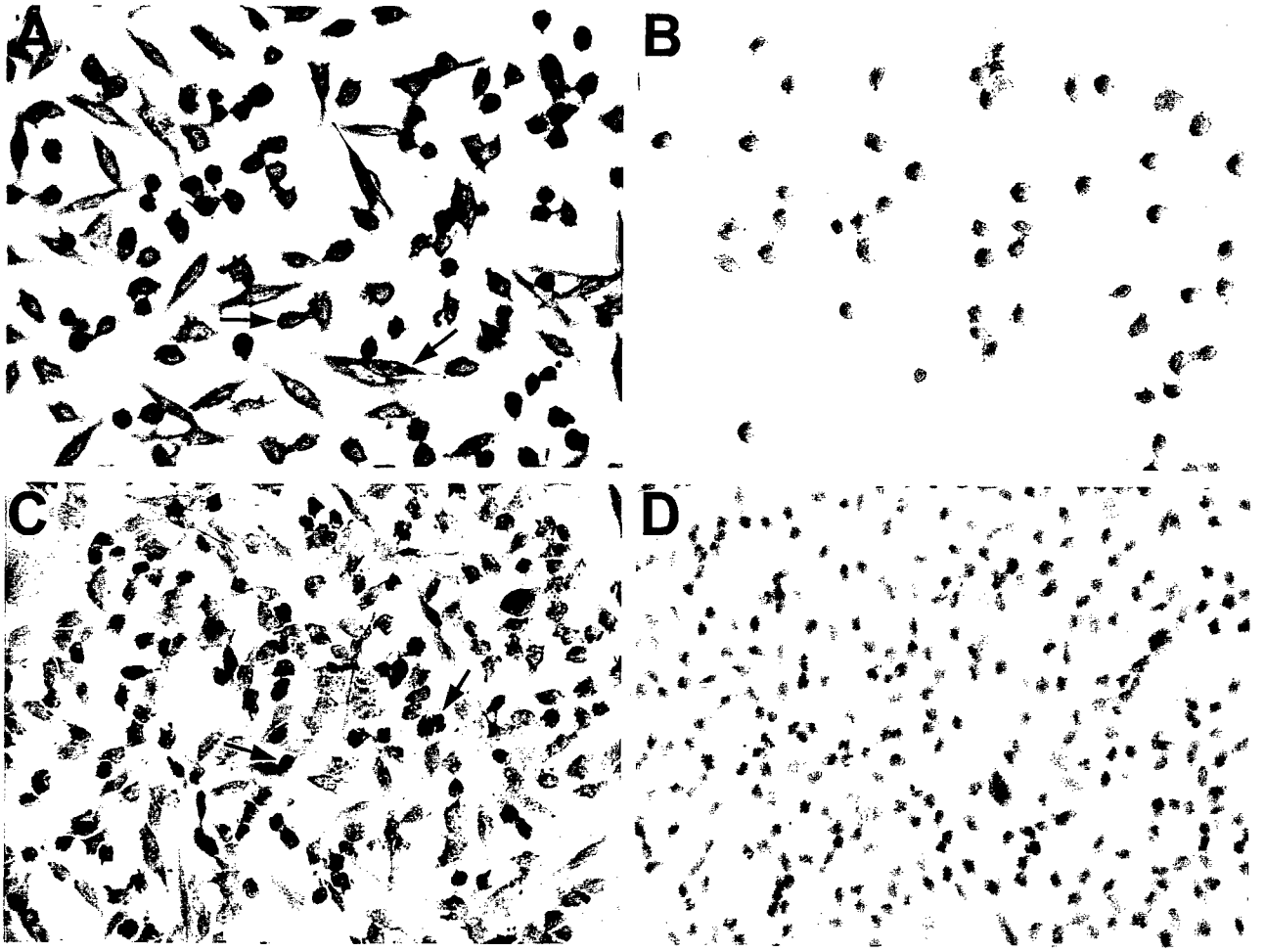

Figure 1

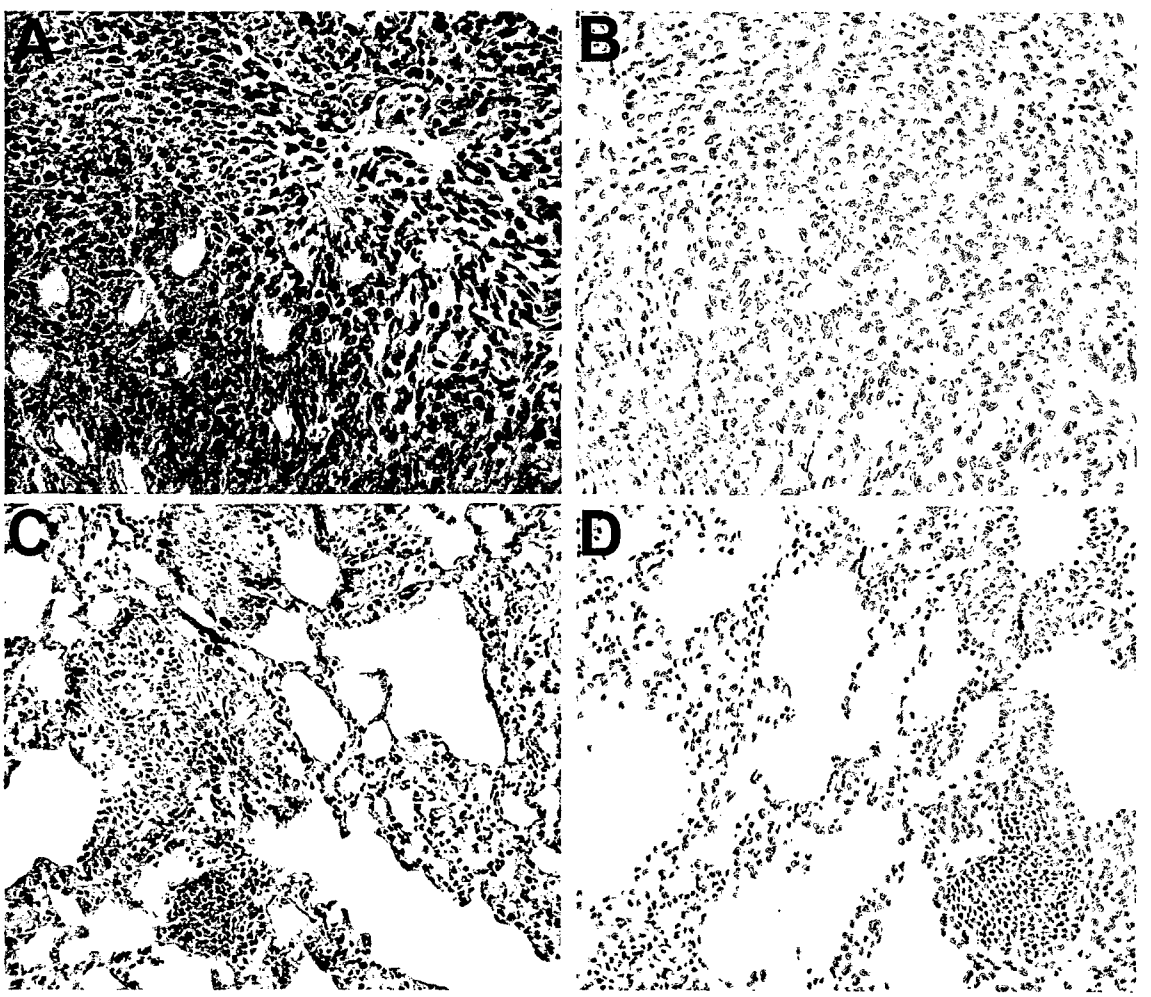

Figure2

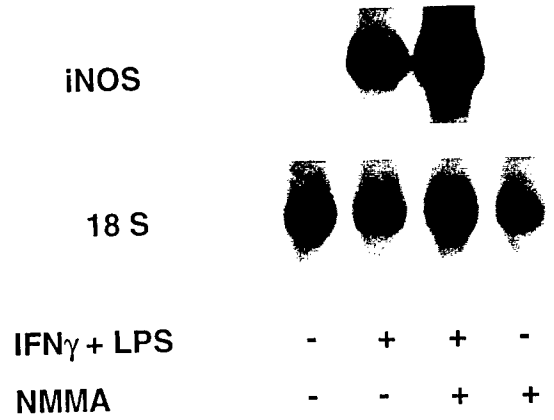

Figure 3A

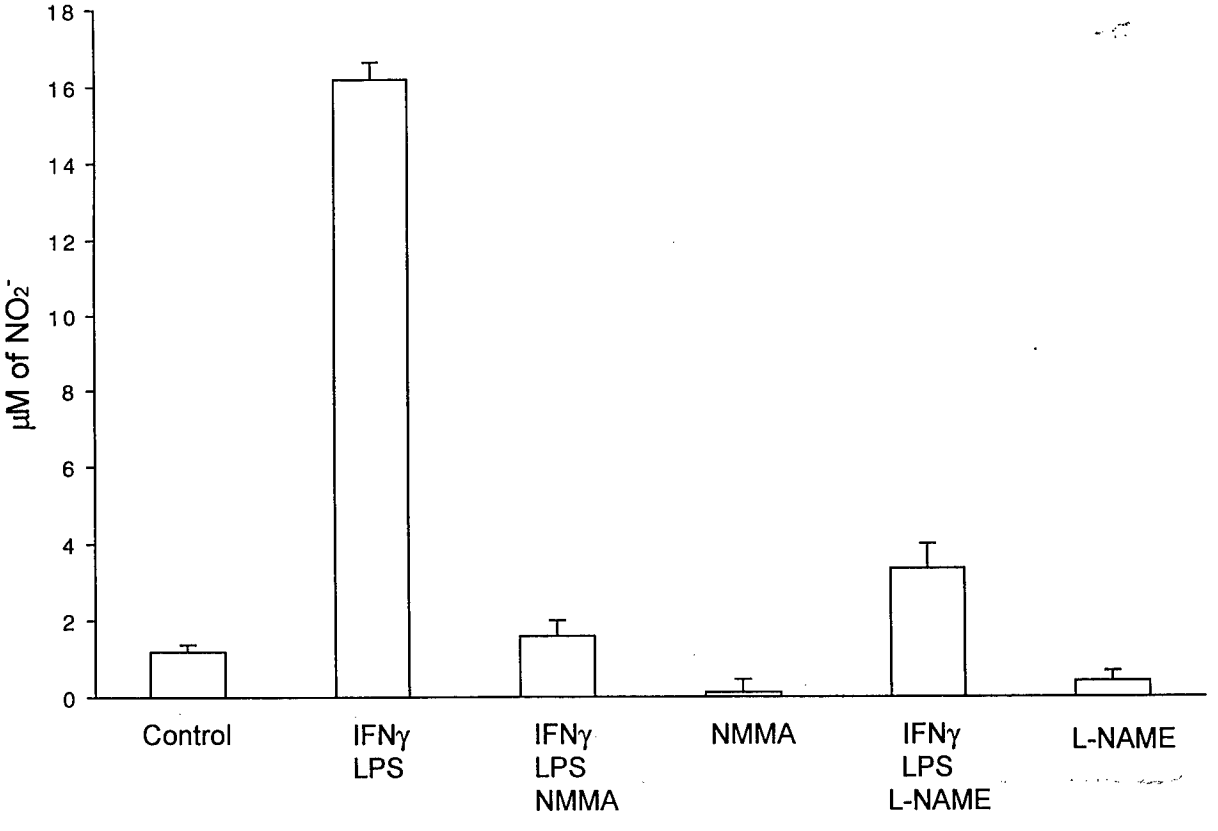

Figure 3B

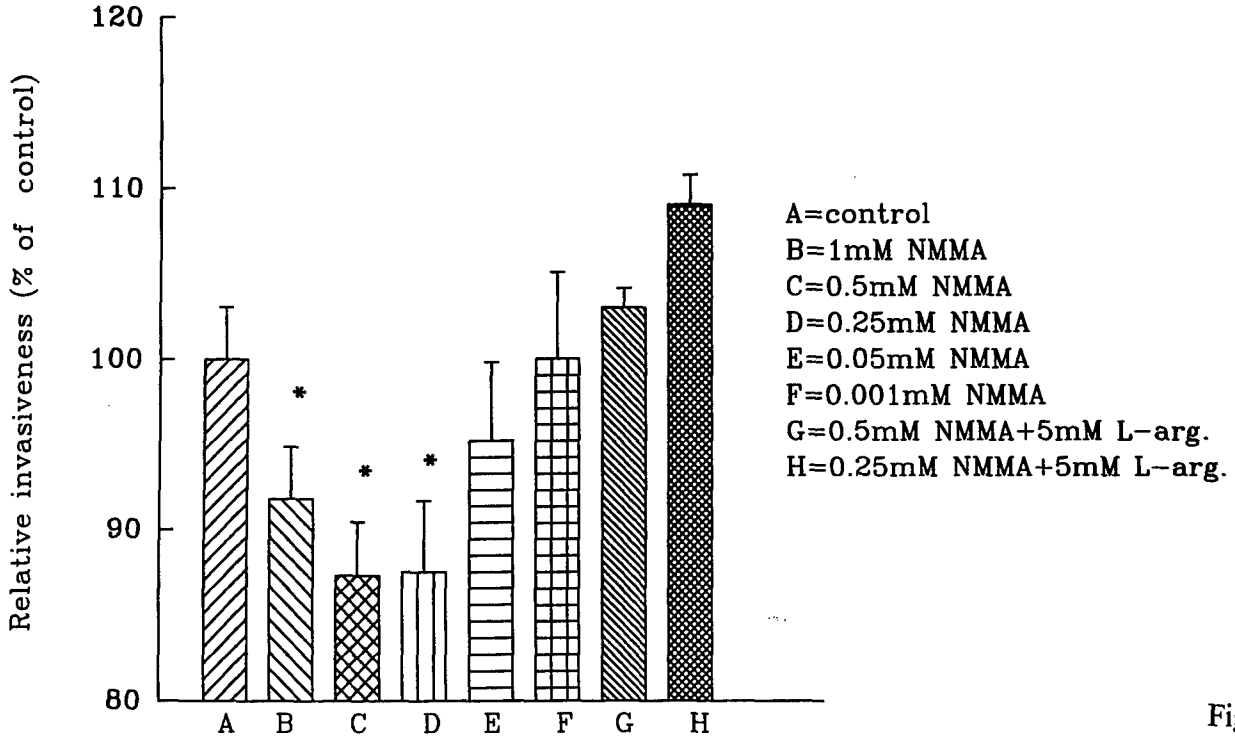

Figure 4

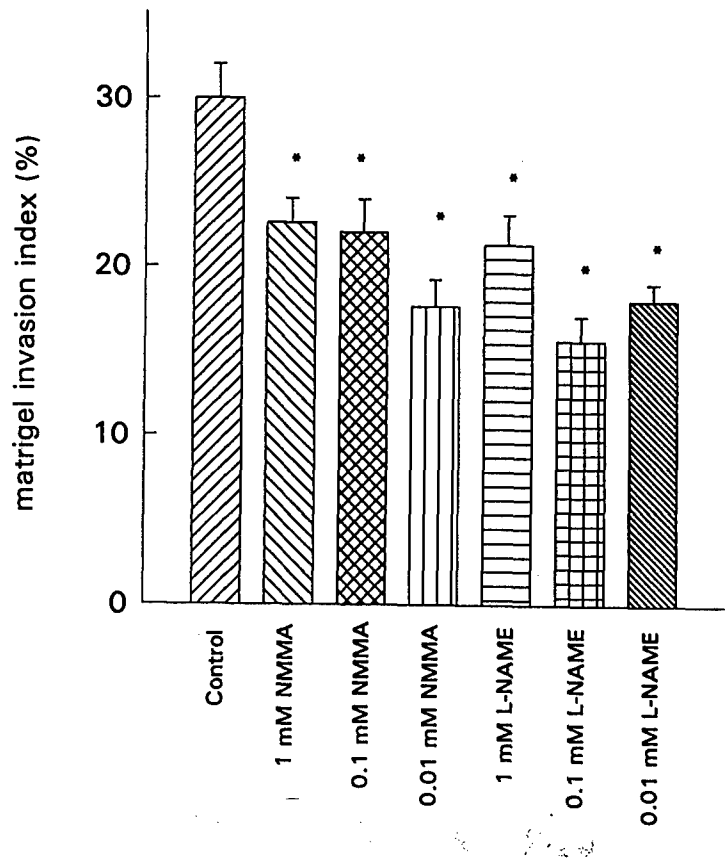

Figure 5

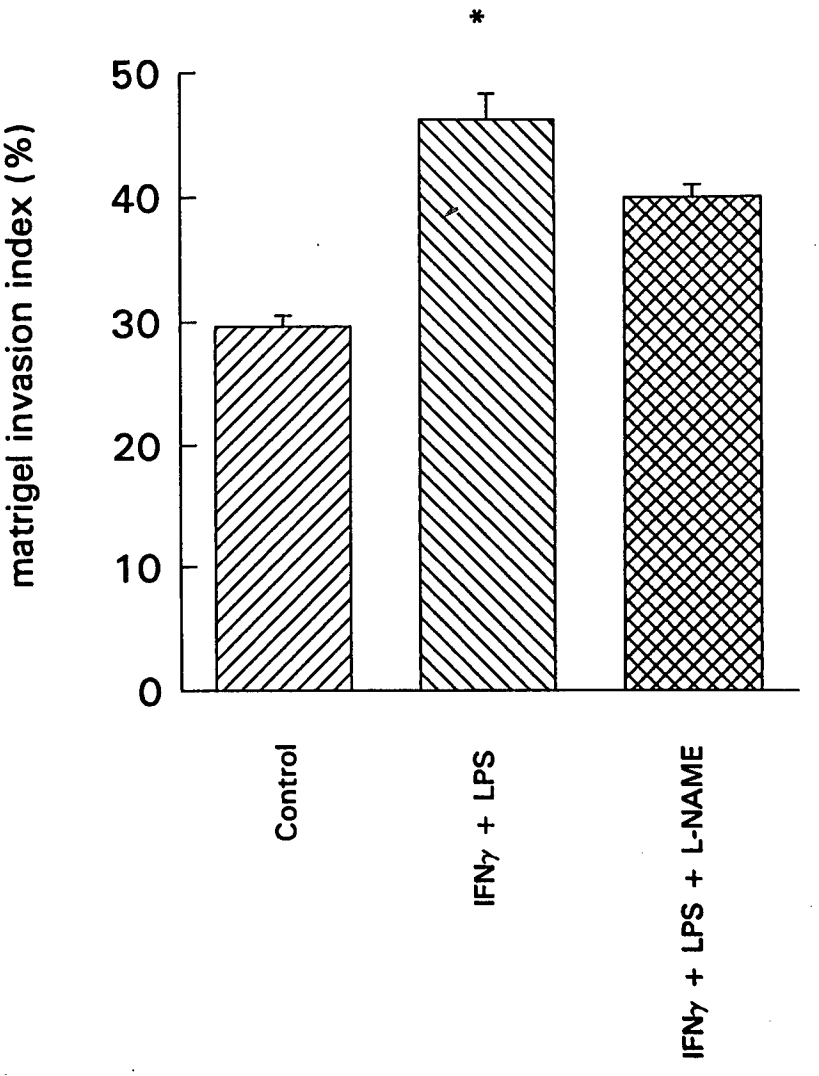
*
50
40
30
20
10
0
matrigel invasion index (%)
Control
IFNγ + LPS
IFNγ + LPS + L-NAME

Figure 6

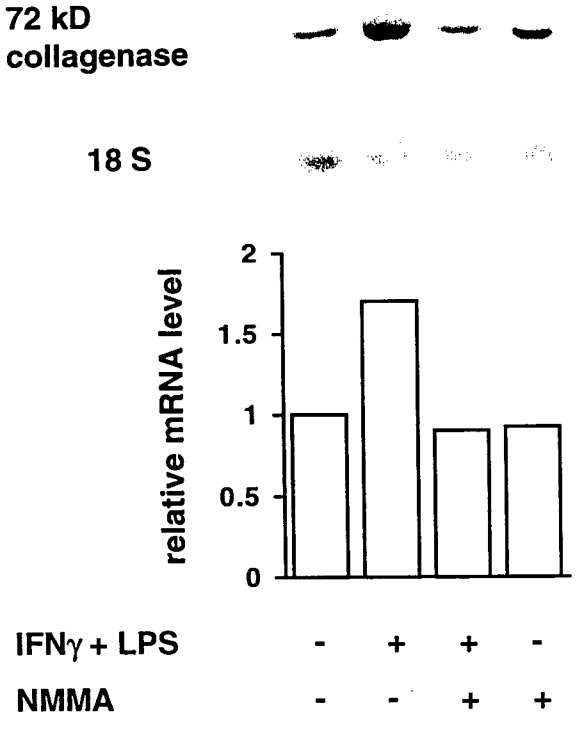

Figure 7A

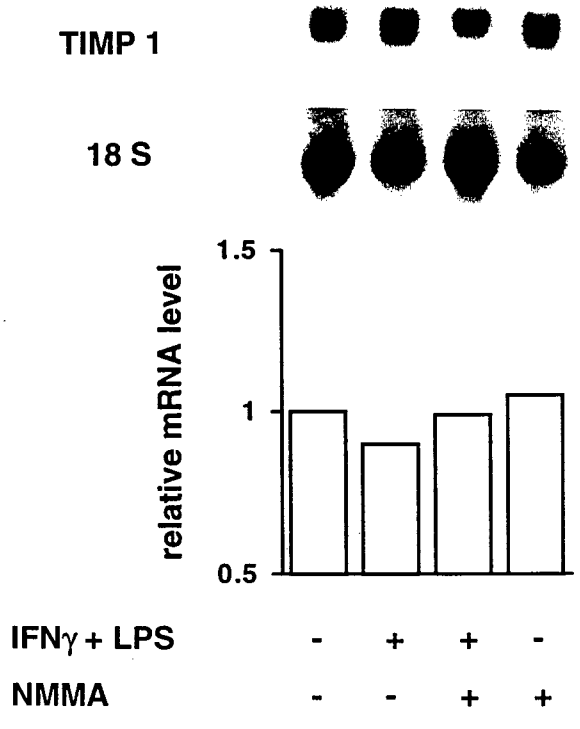

Figure 7B

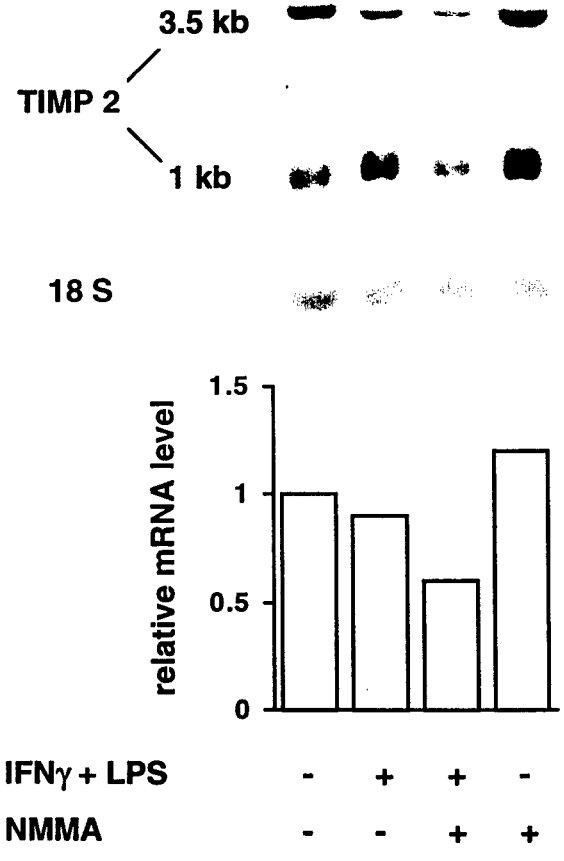

Figure 7C

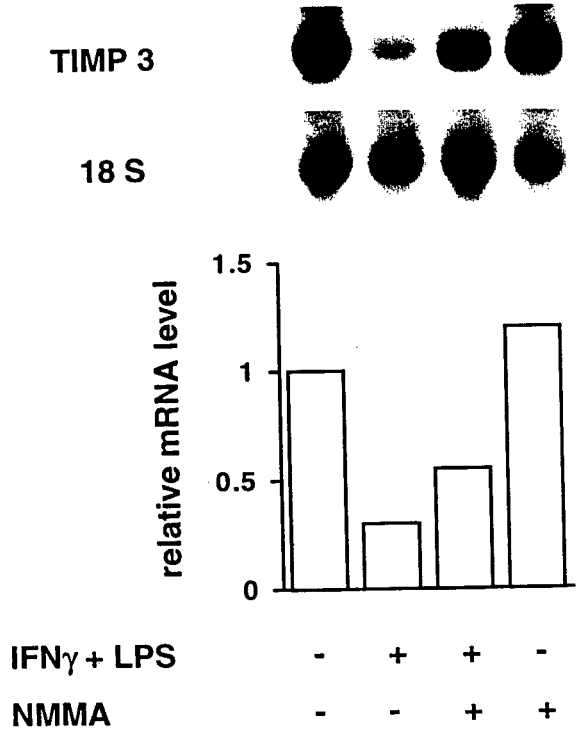

Figure 7D

The Department of Defense
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The Renaissance Hote
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PROCEEDINGS, Volume II

Appendix 5

NITRIC OXIDE (NO)-MEDIATED MAMMARY TUMOR PROGRESSION: ROLE OF NO IN TUMOR CELL INVASIVENESS

Peeyush Lala, Kathleen Hum, Lorraine Jadeski and Amila Orucevic

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There is a positive correlation between the expression of nitric oxide (NO) synthase (NOS) enzymes or NOS activity within the tumor and tumor progression in a number of human tumors: brain tumors, cancers of the reproductive tract and the breast. We observed a similar relationship in a mammary tumor model inclusive of spontaneous and transplanted mammary adenocarcinomas in C3H/HeJ female mice. Ninety percent of retired breeder female mice belonging to this strain develop spontaneous mammary tumors during their life span. We derived two clones from a single spontaneous tumor, which differed widely in their ability for spontaneous lung metastasis from a subcutaneous transplant site: C3L5 was highly metastatic and C10 was poorly metastatic. Immunostaining for NOS enzymes revealed that tumor cells within the spontaneous primary tumors were heterogeneous in their expression of e (endothelial type)-NOS, however, strong and homogeneous eNOS expression was seen in most tumor cells within the spontaneous lung metastases in the same animals. The highly metastatic clone C3L5 showed strong eNOS expression in all cells both *in vitro* as well as *in vivo* within the primary subcutaneous transplants. Spontaneous lung metastases from these transplanted tumors were also strongly positive for eNOS. The poorly metastatic clone C10 showed weak eNOS expression *in vitro*. C3L5 cells also expressed i (inducible type)-NOS when exposed *in vitro* to interferon (IFN)-γ and bacterial lipopolysaccaride (LPS). These findings suggested that NO-producing ability of tumor cells facilitated metastasis in this tumor model. This suggestion was confirmed by treating

Keywords: **Nitric oxide (NO), Invasion; Nitric oxide synthase (NOS), matrix metalloproteases (MMP), Tissue inhibitors of metalloproteases (TIMP)**

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD-17-96-6069 to PKL.

tumor-bearing mice with NO-blocking drugs. Treatment of C3L5 tumor-transplanted mice with NOS inhibitors N^G-methyl-L-arginine (NMMA, given subcutaneously) and N^G-nitro-L-arginine methyl ester (N-NAME, given orally) reduced primary tumor growth and spontaneous lung metastasis, indicating a direct role of NO in tumor progression.

We hypothesized that NO promoted tumor progression in this model by stimulating tumor cell invasiveness and/or tumor angiogenesis. In the present study, we tested whether tumor-derived NO promoted invasiveness of C3L5 cells. Exposure of C3L5 cells to NOS inhibitors (NMMA; L-NAME) reduced their invasiveness as measured in an *in vitro* matrigel invasion assay; this reduction was prevented in the presence of excess L-arginine indicating the NO-specificity of the inhibitor action. Exposure of C3L5 cells to IFN-γ and LPS stimulated NO production *in vitro* (as measured by $NO_2^- + NO_3^-$ levels in the medium) as well as their invasiveness; addition of NOS inhibitors partially abrogated these effects. These results revealed that both native as well as induced NO production by C3L5 cells promoted their invasive function.

We tested whether the NO-mediated stimulation of C3L5 cell invasiveness is due to an alteration in the balance of the synthesis of matrix metalloproteases (MMP-2 and -9) and their natural inhibitors (TIMP-1, 2 and 3); this was achieved by Northern analysis of mRNA expression of these molecules under different experimental conditions. Exposure of C3L5 cells to NOS inhibitor NMMA upregulated TIMP-2 and TIMP-3. Exposure of these cells to IFN-γ and LPS, which induced additional NO production, upregulated MMP-2 and down-regulated TIMP-3; addition of NMMA under these conditions restored MMP-2 expression to normal levels and partially restored TIMP-3 expression. These results revealed that native endogenous NO promoted C3L5 tumor cell invasiveness by down-regulating TIMP-2 and TIMP-3; that IFN-γ and LPS mediated stimulation of invasiveness was partially explained by additional NO induction, which upregulated MMP-2 and down regulated TIMP-3.

In conclusion, tumor-derived NO promotes tumor progression in C3H/HeJ mammary tumors. This is, at least in part, due to a stimulation of tumor cell invasiveness because of an alteration in the balance between the synthesis of MMP-2 and its inhibitors TIMP-2 and TIMP-3.

Proceedings

Eighty-ninth Annual Meeting

American Association for Cancer Research

March 28-April 1, 1998

New Orleans, LA
Page 212, Volume 39 March 1998

#1450 Nitric oxide synthase expression promotes murine mammary tumor progression and metastasis. Hum, K. and Lala, P.K. *Dept. of Anatomy & Cell Biology, The University of Western Ontario, London, Ontario, Canada, N6A 5C1.*

C3H/HeJ female mice bearing transplants of the highly metastatic mammary tumor cell line C3L5 (clonally derived from a spontaneous C3H/HeJ mammary tumor) experienced a decrease in both primary tumor growth and spontaneous lung metastases after treatment with nitric oxide (NO) inhibitors. This led us to investigate the possible relationship between the expression of NO synthases (NOS), as detected with immunohistochemistry, and tumor progression and metastasis. Spontaneous mammary tumors in C3H/HeJ female retired breeder mice consisted of pseudoacinar tumor cell clusters that were heterogeneous for endothelial (e) NOS whereas their lung metastases were homogeneously eNOS positive. Macrophages in the tumor stroma expressed inducible (i) NOS. *In vitro*, C3L5 cells were strongly eNOS positive and could be induced to express iNOS when precultured with LPS and INF-γ. On the other hand, C10 cells, a poorly metastatic mammary tumor cell line clonally derived from the same spontaneous tumor as the C3L5 line, exhibited weak eNOS positivity. *In vivo*, S.C. transplanted C3L5 primary tumors and their spontaneous lung metastases were largely eNOS positive, whereas C10 tumors were more heterogeneous and largely eNOS negative. These results suggest that the expression of eNOS in tumor cells promotes tumor progression in this mammary tumor model. NO-mediated stimulation of tumor cell invasiveness and tumor angiogenesis have been identified as two underlying mechanisms. (Supported by US AMRAA, Grant #DMAD 17-96-1-6096)

Proceedings

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New Orleans, LA
Page 378, Volume 39 March 1998

#2574 Role of nitric oxide in murine mammary tumor angiogenesis.
Jadeski, L., Lala, P.K. *Department of Anatomy and Cell Biology, University of Western Ontario, London, Ontario, Canada N6A-5C1.*

Using a murine breast cancer model, our laboratory has previously found a positive correlation between the expression of nitric oxide synthase (NOS) and tumor progression; treatment with inhibitors of NOS, N^G-Methyl-L-Arginine (NMMA) and N^G-Nitro-L-Arginine methyl ester (L-NAME), had antitumor and antimetastatic effects that were partly attributed to reduced tumor cell invasiveness. In the present study we examined the role of NO in angiogenesis induced by a highly metastatic murine mammary adenocarcinoma cell line, C3L5, which expresses eNOS *in vitro* and *in vivo*, and iNOS *in vitro* upon stimulation with LPS and IFN-γ. Female C3H/HeJ mice received subcutaneous implants of 1) 5×10^4 C3L5 cells suspended in growth factor-reduced Matrigel, or 2) Matrigel alone; L-NAME and D-NAME (inactive enantiomer) were subsequently administered for 14 days using osmotic minipumps. Immediately after sacrifice, implants were removed, fixed with 10% buffered formalin, processed for paraffin embedding, sectioned and stained with Masson's trichrome or analyzed immunohistochemically for vonWillbrand factor (vWF) and CD31 antigens; neovascularization was then quantified (i.e., maximum number of blood vessels per field using 160X magnification) within the implants. Measurable angiogenesis occurred only in implants inclusive of tumor cells; tumor-induced neovascularization was reduced ($P < 0.001$) in L-NAME-treated mice (42.15 ± 5.359) relative to those receiving D-NAME (83.24 ± 10.22), in conjunction with a reduction in the tumor cell population size within implants. These data suggest that antitumor effects of L-NAME were partly mediated by reduced tumor angiogenesis. (Supported by US AMRAA, Grant #DMAD 17–96-6096)

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 18 Jun 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request for Change in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for Grants DAMD17-96-1-6096 and DAMD17-98-2-8019. Request the limited distribution statements for the Accession Documents listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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